AN ABSTRACT OF THE THESIS OF

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Title: THE ANALYSES OF THE MONOSACCHARIDES OF CELLULOSIC HYDROLYZATES BY LIQUID CHROMATOGRAPHY

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Murray L. Laver

A routine method has been developed for the hydrolysis of cellulosic materials. The method involves the dissolution of 350 mg of material in 3 ml of 77% sulfuric acid followed by dilution of the sample to 128 ml with distilled deionized water. The dilute acid solution is refluxed for 4.5 hours, filtered and diluted to 250 ml in a volumetric flask. A 5-ml aliquot of this solution is then added to a 10-ml volumetric flask along with a carefully weighed amount of internal standard, usually D-lyxose, and water is added to 10 ml. A 10-µl sample of this final solution is injected directly into a liquid chromatograph.

A liquid chromatographic method for the quantitative analyses of the monosaccharides released on the acid hydrolysis of cellulosic materials was investigated. The sugars

L-rhamnose, D-mannose, L-arabinose, D-galactose, D-xylose and D-glucose were effectively separated on a column of anion-exchange resin using 0.13 M potassium borate buffer as the eluting solvent.

The sugars were detected after elution from the column with a reagent containing cupric sulfate and dipotassium 2,2'-bicinchoninate. The monosaccharides reduced the cupric ion to the cuprous state and the dipotassium 2,2'-bicinchoninate complexed with the resulting cuprous ion to form a lavender-colored solution whose absorptivity was measured at 546 nm. Quantitative results were obtained by using D-lyxose as an internal standard. Heating was required to develop the color and it was found that heating time considerably affected the amount of color developed. Thus it became necessary to carefully standardize the length of time the colored complex was heated.

Other methods of monosaccharide separation, including acetonitrile-water solvents, were studied by liquid chromatography. However, the separation of the particular monosaccharides released on hydrolysis of cellulosic materials was incomplete. The advantages of the borate buffer system are : (1) the acid hydrolyzate does not have to be neutralized, it can be diluted and injected directly into the instrument; (2) the hydrolyzate does not have to be concentrated as a consequence of neutralization; (3) the free forms of

the monosaccharides can be separated so no derivatization is necessary; (4) the monosaccharides are well separated and thus each sugar can be quantitatively analyzed. Because of these advantages, particularily the ease of sample preparation, the borate buffer method allows for routine analyses of the monosaccharides in cellulosic hydrolyzates.

A cotton cellulose was purified and used as a standard to establish the method. A yield of 99.9% D-glucose was obtained for this standard cellulose. Several commercial pulp samples and a thermomechanical pulp were also analyzed. The results for the commercial pulps showed D-glucose quantities ranging from 80.0% to 99.6%, D-xylose quantities ranging from 0.4% to 5.9%, and D-mannose quantities ranging from 0.1% to 1.0%. The thermomechanical pulp analyzed for 50.6% D-glucose and 17.1% D-mannose.

The Analyses of the Monosaccharides of Cellulosic Hydrolyzates by Liquid Chromatography

by

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APPROVED:

Redacted for privacy

Associate Professor of Forest Products Chemistry
in charge of major

Redacted for privacy

Head of Department of Forest Products

Redacted for privacy

Dean of Graduate School

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I would like to dedicate this thesis in memory of my father, Chen-I Ni, who also was an organic chemist. I trust that this study will assist in fulfilling his career in the field of carbohydrates.

Special thanks to Mr. Kung Shih for his enthusiastic help in analyzing the experimental data in this study.

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I. INTRODUCTION

Historically, trees have been of great use to mankind. In this time of energy and material shortages trees are attracting more attention than ever because they are renewable.

Cellulose, a polymer, is known to constitute the skeleton of trees, and supplies most of the supporting strength. D-glucose is the monomeric building unit of the cellulose polymer. It is theoretically the only constituent of cellulose and the β 1→4 linked D-anhydroglucose units form a straight, long chain polymer. Hydrogen bonding between the molecular cellulose polymers constitute forces of attraction which, at least in part, bind the individual cellulose molecules into microfibrils and fibers.

However, D-glucose is not the only simple sugar present in trees in the form of polymers. D-mannose, D-galactose, D-xylose, L-arabinose and sometimes L-rhamnose are also found in the acid hydrolyzates of wood. Due to the demand for better quality in certain wood products, such as paper and films, it became desirable to know how much of each monosaccharide sugar is present in wood and wood products.

At present it is more important than ever to know how much glucose can be obtained from the hydrolysis of wood and wood products. D-glucose is a potential energy source because it can be fermented to ethanol. For these reasons, experiments have been carried out to find a faster, easier routine method for the hydrolysis of wood and wood products and to quantitatively analyze for the monosaccharides released.

In this laboratory gas-liquid chromatography and high performance liquid chromatography have been used to quantitatively analyze the monosaccharides in wood hydrolyzates. This study involves the use of different techniques, instrumental conditions and the parameters of time and temperature.

The specific objectives of the present work were: 1) to develop a routine method for the hydrolysis of wood and wood products; 2) to develop a routine method for the quantitative analysis of the monosaccharides released on the hydrolysis of wood and wood products; 3) to apply these routine methods to the analyses of selected wood products, especially pulps prepared by chemical means, and thermomechanical pulps.

II. HISTORICAL REVIEW

The hydrolysis of cellulose, which may come from cotton, wood, bark and other resources, had been tried for years. However, none was successful until 1921, when Monier-Williams (24) developed a method for the hydrolysis of cellulose which involved dissolution in concentrated 72.0% sulfuric acid. This method has provided the basis for most of the later developments and improvements.

In his original procedure, Monier-Williams obtained a dark-colored, viscous solution from the dissolution of cotton wool in 72.0% sulfuric acid by allowing it to remain for one week at room temperature. The solution was then diluted to 1.2% sulfuric acid with water, and refluxed for 15 hours. Solid barium carbonate was used to neutralize the solution. After filtration and concentration, D-glucose was crystallized from a methanolic solution of the hydrolyzate.

A widely used modification of the Monier-Williams technique is that of Saeman and co-workers (29,30,31), who used 72.0% sulfuric acid to dissolve the sample for 1.0 hour at 30°. However, in this modification it was found that it is often difficult to dissolve wood pulp completely. As a consequence, some of the remaining higher molecular weight polysaccharides may precipitate after dilution of the acid with water. A misty or cloudy solution shows incomplete dissolution, which will provide a low yield on analysis.

In 1960, Jeffery, Partlow and Polglase (17) found that using 77.0% sulfuric acid could avoid this problem and get better dissolution. Giertz (13) had also shown that evacuation of the flask several times also helped to promote rapid solution of the pulp in the acid. Saeman, Moore and Millet (31) and Jeffery, Partlow and Polglase (17) diluted the hydrolyzate to 4% sulfuric acid and heated it at the boiling point for 4 hr. The former used 15 psi steam, the latter used a boiling water bath. However, Saeman, Moore, and Millet (31) found that these two heating methods made no difference. It was also found that 3% sulfuric acid and a longer reflux time provided a slower reaction that could be more easily controlled (21).

Paper chromatographic and gas-liquid chromatographic analyses require neutralization of the sample. There are several ways this can be accomplished. It can be done with saturated aqueous barium hydroxide, solid barium carbonate or ion exchange resins. With ion exchange resins it is difficult to quantitatively recover the carbohydrates for some of them might stay on the resin or be converted to others which can be very difficult to analyze. Barium carbonate is not a good method because it is difficult to dissolve completely. Hence, too much is usually added which later dissolves leaving an excess of barium ions in the solution. These are difficult to remove and create difficulties for the chromatographic analyses used later.

Saturated aqueous barium hydroxide usually is the best method because it contains only a very small amount of barium ions. A heterogeneous reaction that may involve excess reagent and absorption can be avoided, and also the pH can be better controlled. The hydrolyzate is usually neutralized to pH 5.0. After neutralization and centrifugation to remove the resulting barium sulfate precipitate the solution is very dilute in sugars. Therefore, the solution has to be concentrated. Concentration of the solution should be performed under vacuum at a temperature below 60°. A rotary evaporator is best suited for this purpose. Alkaline conditions created by accumulation of the dissolved barium hydroxide during evaporation must be avoided because the types of sugars and their properties may be altered by alkalinity. Monier-Williams (24) added methyl red to the solution as an indicator, and the solution was kept neutral by repeated additions of 0.1 N sulfuric acid. The action of alkali follows three general courses (4): 1) isomerization, mainly at the reducing end of the molecule, 2) fragmentation due to alkaline oxidation and, 3) internal oxidation and reduction. In the usual cases, excess alkalinity was avoided by neutralization to pH 5.0 and careful concentration of the hydrolyzates at 60°.

Chromatography is a good way to separate mixtures. Sugar mixtures of hydrolyzates from the hydrolyses of wood and cellulosic materials may contain a variety of monosaccharides such as L-rhamnose, L-arabinose, D-mannose,

D-xylose, D-galactose and D-glucose.

These monosaccharides can be separated successfully by various chromatographic methods. Saeman and co-workers (30,31) used Whatman No.1 paper and the application of a uniform streak of sample. The hydrolyzates can also be analyzed quantitatively. Jeffery, Partlow, and Polglase (17) employed a reflectance measurement on a colored chromatogram. However, the techniques involved were complex and the large amounts of glucose in pulp samples limited quantitative resolution of the other sugars. Thin-layer chromatographic methods have also been used for the detection of sugars in hydrolyzates (39). Although both qualitative and quantitative results can be obtained, the procedure is time consuming and the quantitative results are not too reproducible.

Gas-liquid chromatography can be used for the analyses of many closely related compounds. However, the separation of carbohydrates has proved difficult because of their heat sensitivity and lack of volatility, which are the basic requirements for gas-liquid chromatographic analyses. These problems can be solved by converting the parent sugars or their methyl glycosides to the more volatile O-methyl ether, acetate or trimethylsilyl ether derivatives. The trimethylsilyl ether method, which can give quantitative results (1,32) has been applied to wood pulps (6). The formation of the derivative is rapid and convenient, but there are limitations. Water reacts with the reagents and extensive drying

techniques are required. Furthermore, interpretation of the chromatograms is complicated because equilibria of the reducing sugars generate the anomeric forms and even the ring isomers, which are difficult to resolve. The results are, therefore, dependent on measurement of the individual equilibrium isomers, primarily the anomeric pair, and the reproducibility of their ratios.

Gunner, Jones, and Perry (15) developed a method whereby single peaks can be obtained by converting the sugars to their corresponding alditol acetates. A method based on this approach was developed for quantitative analysis (33). It also has been applied to wood pulp samples (8,5). This method is excellent, but the formation of these derivatives is lengthy and complex and also requires care for quantitative analysis. An internal standard has been used to eliminate the uncertainties due to the difficulties of obtaining absolute reproduction of the operating conditions. Myo-inositol works well as the internal standard in the alditol acetate procedure. Borchardt and Piper (5) determined the relationship between the area under the peaks of the alditol acetates of glucose, mannose, galactose, arabinose and xylose. They used these area ratios in their calculations of the amount of monosaccharides in several pulp hydrolyzate samples.

The relatively recent improvements in equipment for liquid chromatography and, in particular, high-performance

liquid chromatography (HPLC) has resulted in methodology aimed at separating the carbohydrates without derivatization. A bibliography of papers related to the separation of carbohydrates by liquid chromatography was published by Johnson (18) in 1977 and numerous papers have appeared since. There are too many papers to be thoroughly reviewed in this thesis and only those considered pertinent to the research will be mentioned.

In 1974 Belue and McGinnis (2) showed that water-soluble wood polysaccharides could be separated on Bio-Glas (granular porous glass) and Bio-Gel P (polyacrylamide) packed columns using high-pressure liquid chromatography with water as the solvent system. Palmer (28) in 1975 showed that selected monosaccharides could be separated on a Waters Associates "μ BONDAPAK/carbohydrate" column using various proportions of water:acetonitrile as eluant. Although this system separated the pentoses quite well, the hexoses were poorly resolved and so the system was less than desirable for wood hydrolyzates. McGinnis and Fang published two papers (22, 23) concerned with the separation of substituted carbohydrates by high-performance liquid chromatography. However, the preparation of derivatives is lengthy and not always quantitative. If derivatives have to be prepared then liquid chromatography has little or no advantage over gas-liquid chromatography and the alditol acetate method.

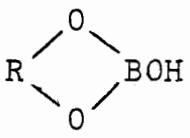
Several of the equipment manufacturers (16,38,36) have published data concerning the separation of carbohydrates on prepacked columns. However, none of these columns and solvent systems completely resolve the monosaccharides resulting from the hydrolysis of wood and wood products. A recent brochure by Bio-Rad Laboratories (3) describes the use of their Aminex HPX-85 heavy metal form cation exchange column using water as the eluant. A spectrum of a wood pulp hydrolyzate is shown in which cellobiose, glucose, xylose, and mannose are well resolved. A spectrum of known sugars is also shown in which glucose, xylose, and galactose are well resolved and arabinose and mannose are not separated. However, this system could be useful for pulp hydrolyzates because they usually contain little or no arabinose so the interference with the mannose should be slight.

Ladisch, Huebner, and Tsao (19) and Ladisch and Tsao (20) have used cation-exchange resins to separate some carbohydrates. Their system also utilizes water as eluant and is thus quite simple. However, they have not been able, as yet, to separate the monosaccharides sufficiently well for quantitative analyses.

Sinner, Simatupang, and Dietrichs (34) in 1975 published a paper describing the complete separation of glucose, mannose, arabinose, galactose, xylose and glucose using borate complex ion exchange liquid chromatography. The use of

improved anion-exchange resins considerably shortened the separation time of the sugars. The authors utilized aqueous potassium borate buffer as the eluting solvent for liquid chromatography.

The separation of monosaccharides by complexing with borate ions is well established in paper electrophoresis (9, 37). The conductivity of certain polyhydroxy compounds in borate solution has been utilized to separate neutral carbohydrates by paper electrophoresis. The esters and complexes between boric acid and borate ions respectively, and polyhydroxy compounds can be formulated as follows:



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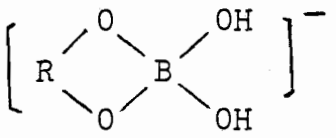

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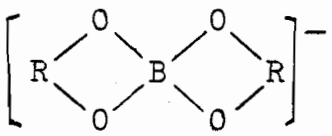
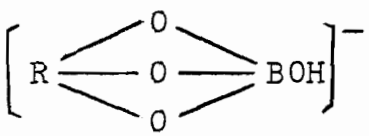


III



IV

The ionic species II, III, and IV, migrate during electrophoresis. It has been suggested (37) that the borate ion is able to complex with those compounds in which the oxygen

atoms of at least two hydroxyl groups are separated by, or can approach each other, to a distance of 2.4 Å. Although no electrical current is used in the anion exchange column chromatographic method outlined by Sinner, Simatupang and Dietrichs (34), the ability of the various monosaccharides to complex with the borate ions in the eluant buffer is critical. The ease of complex formation and the stability of the complexes is undoubtedly due to the stereochemistry of the sugar molecules with respect to their hydroxyl groups. The monosaccharides are thus separated in relation to their stereochemistry. This will be discussed more fully in the discussion section of this thesis.

Monosaccharides, when they elute from the column, are colorless. Therefore, a chemical must be mixed with the eluant stream which reacts with the monosaccharides to give color which can be measured. Sinner, Simatupang and Dietrichs (34) first used an orcinol reagent in concentrated sulfuric acid. However, this proved to be a very corrosive reagent and caused problems with the equipment in routine work. In a later publication Sinner and Puls (35) used a reagent which included copper sulfate and disodium 2,2'-bicinchoninate. This reagent had been previously used by Mopper and Gindler (25). The reaction of this dye with sugars will be discussed in the discussion section of this thesis.

Friberg, Barnes, and Meyers (10) have modified the

original system of Sinner, Simatupang and Dietrichs (34) to include mixers and good solvent pumps to provide a stable baseline. However, no work has been reported previously concerning the use of internal standards and instrument factors to ensure reproducible and quantitative results.

III. EXPERIMENTAL

A. High-Performance Liquid Chromatography

1. Acetonitrile-Water System

A Waters Associates High-performance Liquid Chromatographic System was used. There were two liquid chromatography pumps Model 6000A, the differential refractometer indicator was Model R401 and the column was a prepacked carbohydrate column of the microbondapack type [μ carbohydrate (P/N 84038)].

The eluting solvent was acetonitrile-water 85:15 v/v. Myo-inositol was used as an internal standard. Solutions of standard sugars and myo-inositol were prepared such that the weight ratios 0.5, 0.75, 1.0, 1.25, 1.5, 1.75, 2.0, 2.25, 2.5, 2.75 and 3.0 could be used to determine instrument " K " factors. Typical conditions for the runs were: flow rate 1.7 ml/min, attenuation on the differential refractometer 8X. A Hewlett Packard integrator Model HP 3380A was used to integrate the peak areas. The conditions of integrator were: area %, start delay 2 min (avoids integration of solvent water peak), stop timer 20 min (shuts off automatically), area reject off, chart speed 0.5 cm/min, chart auto, slope sensitivity 1 mv/min, attenuation 32.

2. Borate Buffer System

a. Borate Buffer Preparation The eluant solvent borate buffer was prepared by dissolving potassium hydroxide (51.05 g) in distilled deionized water (3 liters) followed by the dissolution of boric acid (112.48 g). Additional distilled deionized water (500 ml) was added to yield a solution 0.52 M in boric acid (H_3BO_3) or 0.13 M in potassium borate ($K_2B_4O_7$) in accordance with the balanced chemical equation:

$$2\ KOH + 4\ H_3BO_3 \rightarrow K_2B_4O_7 + 7\ H_2O$$

The pH of the buffer was 8.9. This solution was vacuum filtered through a 47 mm diameter, 0.45 μm poresize filter. The eluant was pumped through the column at a flow rate of 0.2 ml/min. The temperature of the column was 57.5° which was maintained by pumping water of this temperature through a water jacket. A magnetic stirrer was used to keep the stock solution aggitated while in use.

b. Copper Dye Reagent Preparation

Solution A:

Anhydrous sodium carbonate (215.0 g) was added to 3 liters of distilled deionized water. After complete dissolution, dipotassium 2,2'-bicinchoninate (6.2 g) [$(C_9H_5NCOOK)_2$, F.W. 420.9 , assay 82.4% from Hach Chemical

Co., Ames, Iowa] and 450 ml of distilled deionized water was added with stirring.

Solution B:

L-Aspartic acid (3.7 g), anhydrous sodium carbonate (5.0 g) and cupric sulfate 5-hydrate (1.0 g) was added to 150 ml of distilled deionized water.

Both solution A and solution B are stable for several months. Solution A and solution B are mixed to yield about 3600 ml of final reagent in a volume ratio of 23 parts of solution A to 1 part of solution B. The final solution is stored in a brown bottle and allowed to stand for several hr before use. The solution is vacuum filtered through a 47 mm diameter, 0.45 μm pore size filter. The stored reagent should not stand too long for the color becomes deeper with time.

c. Column Preparation The column used was of precision bore, chromatographic-quality, polished 316 stainless steel tubing. The column, 250 mm long with a 3.2 mm internal diameter and 0.25 inches outside diameter, was equipped with two low, dead column end fittings and two replaceable bed supports constructed of stainless steel frits with a nominal pore size of 2 μm.

The column packing was a Durrum anion-exchange resin described as "Chromex DA-X4-11" (Dionex Corporation, Sunnyvale, CA). About 5 g of the resin was slurried in 1.3 M potassium borate ($K_2B_4O_7$) buffer (50 ml) (ten times as concentrated as the eluant buffer). The resin was recovered on a Whatman No.1 filter paper and washed several times with the borate buffer by vacuum. After washing with distilled deionized water several times, the resin was slurried (about 50% solids) with eluant borate buffer (0.13 M $K_2B_4O_7$). The slurry was transferred with a Pasteur pipette into the column until the column was filled. The column was vibrated with a hand-held vibrator to ensure the complete removal of air bubbles. The column was then connected to the pumps and the eluant borate buffer (0.13 M $K_2B_4O_7$) was pumped through at 0.2 ml/min for 15 min. The inlet end of the column was opened and the clear supernatant above the resin bed was removed with a Pasteur pipette. Additional resin slurry was added until the column was filled again. Eluant buffer was again pumped through the column. The alternate filling of the column and pumping of the eluant was repeated until the resin bed completely filled the column. Eluant was pumped through the column at 0.2 ml/min for a minimum of 2 hr before use.

d. Equipment Two chromatography pumps from Waters Associates Co. were first used to pump borate buffer and

copper dye reagent at flow rates of 0.2 ml/min and 0.5 ml/min respectively. A small pump, Model A-30-S from Eldex Laboratories Inc., Menlo Park, CA. was substituted later for the copper dye reagent. The micrometer dial was adjusted to 1.1 which delivered a flow rate of 0.5 ml/min.

A glass water jacket surrounded the column and water at 57.5° was pumped through the jacket from a Haake Co. circulating water bath.

A T joint combined the borate buffer eluant from the anion exchange column and the copper dye reagent. The mixed solvent streams then passed through 15 meters of teflon tubing (0.8 mm internal diameter) which was immersed in a boiling water bath. The solvent stream was then passed through a Waters Associates Model 440 absorbance monitor. A wavelength of 546 nm was used to detect the sugars after their reaction with the copper dye reagent. An attenuation setting of 0.5 was used with the monitor. A Hewlett Packard Model 3380A integrator was used to give a recorder spectrum of the absorbances and to integrate the areas under the peaks. The integrator settings were: attenuation, 32; slope sensitivity, 0.1 mv/min; chart speed, 0.5 cm/min.

e. Separation of Monosaccharides Due to the complexing of the free sugars with the borate buffer and the resulting exchange with the resin, they eluted from the column in

a specific sequence. After reacting with the copper dye reagent in the boiling water bath, the presence of the sugars could be detected by the absorbance detector. The offset absorbance was set at about 0.400. The detector should be adjusted to zero after the system becomes stablized. The presence of the monosaccharides was shown by increased absorbance on the meter and was recorded on a strip chart recorder by the HP 3380A integrator, from which the areas under the peaks were calculated.

f. Maximum Absorbance and Mechanism of Detection

A 250 ml solution of 8.12% D-glucose was prepared by dissolving 20.3 mg of D-glucose in 250 ml of distilled deionized water. Aliquots of 1 ml, 2 ml, 3 ml, 4 ml and 5 ml of the D-glucose solution were taken and diluted to 50 ml with a mixed solution which was prepared by mixing the copper dye reagent (400 ml) and borate buffer (160 ml). The resulting solution was boiled in a boiling water bath for 15 min. An ACTA III UV-Visible spectrophotometer from Beckman Co. was used to scan the absorbance of each solution from 700 nm to 400 nm. The scan speed was set at 0.5 nm/sec and the chart expansion was set at 100 nm/inch.

g. Effect of Heating Time on the Copper Dye Reagent Alone An aliquot of 3 ml of 77% sulfuric acid was

diluted to 250 ml with distilled deionized water in a volumetric flask. A 5-ml aliquot was taken and diluted to 10 ml with distilled deionized water. From this solution a 10-µl aliquot was added to the mixture of 5 ml of copper dye reagent and 2 ml of borate buffer. Eleven of these solutions were made and one of each was boiled in a boiling water bath for 0, 1, 2, 3, 4, 5, 10, 15, 20, 25 and 30 min. The one that was not boiled (0 min) was used as a reference. The absorbance of each was determined using the UV-Visible spectrophotometer ACTA III from Beckman Co. at a wavelength of 546 nm.

h. Effect of Heating Time on the Reaction of the Copper Dye and the Monosaccharides An aliquot of 3 ml of 77% sulfuric acid was diluted to 250 ml with distilled deionized water in a volumetric flask. Aliquots of 5 ml containing 10.7 mg of D-glucose were then diluted to 10 ml with distilled deionized water. An aliquot of 10 µl was added into the mixture of 5 ml of copper dye reagent and 2 ml of borate buffer. A total of ten such solutions were made. One each of the solutions were boiled for 0, 1, 2, 3, 4, 5, 10, 15, 20, 25 and 30 min. The solution that was not boiled (0 min) was used as a reference. The absorbances were detected at 546 nm by using the Beckman UV-Visible spectrophotometer previously mentioned. Another reference was

prepared by the exact procedure except no monosaccharide (glucose) was added. The absorbance was also detected.

From the results obtained from the previous section, we knew that there was a heating effect on the absorbance of the copper dye reagent alone. The absorbance due to the copper dye alone was subtracted from the absorbances so that the authentic effect of heating on the reaction of the monosaccharides and the copper dye reagent was then obtained.

The same procedures were carried out for D-lyxose.

i. Obedience of Beer's Law An aliquot of 3 ml of 77% sulfuric acid was diluted to 250 ml with distilled deionized water in a volumetric flask. Twenty 5 ml aliquots were taken and weights of glucose ranging from 2.0 mg to 65.0 mg were added. The solutions were diluted to 10 ml with distilled deionized water in volumetric flasks. From these solutions, 10 µl aliquots were added into mixtures of 2 ml of borate buffer and 5 ml of copper dye reagent. These solutions were boiled in a boiling water bath for 5 min. Absorbances were measured by the Beckman ACTA III UV-Visible spectrophotometer at 546 nm. The reference used was the mixture of 2 ml of borate buffer and 5 ml of copper dye reagent to which no monosaccharides had been added and which had not been boiled. The absorbance detected included the effect of heating on the copper dye reagent itself.

j. Internal Standards and Standard Curves Many monosaccharides and disaccharides were tried to see if they met the three basic requirements for a good internal standard. 1) Similar chemical and physical properties that the components have but do not react with them nor interfere with any reaction that the components might undergo. 2) Its peak is in the portion of the spectrum where no peaks from the components are present, that is, it does not exist in the sample been analyzed and the retention time is suitable and does not interfere with the separation. 3) Easily obtained and cheap. It was not until later that the choice of disaccharides was discarded because they could be hydrolyzed during the run and interfere with the actual components. Rhamnose and lyxose were found to be suitable. However, there was still a very small chance that rhamnose might be found in wood hydrolyzates. Lyxose was then chosen as the internal standard. A set of standard solutions with known weight ratios of glucose to lyxose were prepared. The ratio range for glucose was from 0.235 to 2.02 because glucose is always the main sugar in the acid hydrolyzates. Its ratio might vary dramatically. A set of standard solutions with known weight ratios of mannose to lyxose was also prapared. The ratio range for mannose was from 0.11 to 0.60 because it is usually found in small amounts in the acid hydrolyzates.

A simulated solution was prepared by diluting a 3 ml-aliquot of 77% sulfuric acid to 250 ml in a volumetric flask with distilled deionized water. An exact replication of the sulfuric acid concentration resulting from the hydrolysis of an actual sample was found to be critical. Aliquots of 10 µl each of the standard solutions were injected into the HPLC. In this way, ratios of the weight of D-glucose:weight of D-lyxose and the corresponding ratios of the area of D-glucose peak:area of D-lyxose peak were obtained. The area ratios of D-mannose:D-lyxose were similarily obtained.

B. Hydrolyses of Polysaccharide Samples

1. Dissolution and Primary Hydrolysis

Polysaccharide samples which had been ground in a Wiley mill to pass a 40 mesh screen, and which had been oven dried at 105° (350 mg) were dissolved in 3 ml of 77% sulfuric acid at room temperature. Occasional application of vacuum to the reaction flask helped the dissolution of the sample. However, it was found that the most effective way was to keep stirring with a glass rod. "Squelching" the unsoaked sample often made the dissolution go faster. The dissolution was completed within 30-60 min. Complete dissolution of samples could be checked by examination of the solutions to see if

they were viscous or not. A non-viscous solution meant the dissolution was complete. With purified cotton cellulose a light-brown color resulted. With other polysaccharide samples such as pulps, a brown to black color developed.

2. Total Hydrolysis

The solution was then transferred to a 250-ml round bottomed flask with distilled deionized water (50 ml). After mixing well, another portion of distilled deionized water (75 ml) was added to make a total volumn of 128 ml and a 2.84% concentration of sulfuric acid. It is then ready to be refluxed. Because the reflux time was closely related to the recovery yield, 4.5 hr was chosen as a standard refluxing time (21). Procedures used in this laboratory are outlined in chart 1 (page 24).

3. Kinetic Study of the Hydrolysis of Pure Cellulose

Cotton was purchased from the drug store and purified by the procedure outlined by Corbett (7). The cotton was extracted with choroform for 19 hr (Soxhlet apparatus). The extracted cotton was boiled vigorously in 1% sodium hydroxide for 8 hr by using a 3000-ml three necked round-bottomed flask

Oven dried, milled
polysaccharide sample (350 mg)

↓ Dissolve in 77% sulfuric acid (3 ml) for 1 hr

Viscous paste solution

↓ Dilute to 128 ml with distilled deionized water

Total of 128 ml of 3% sulfuric acid solution in 250-ml round-bottomed flask

↓ Reflux for 4.5 hr and cool. Filter through Whatman No.1 filter paper and wash well with distilled deionized water

Residue on the filter paper

↓ Dry in oven (105°) for 2 hr

Acid insoluble lignin

Filtrate

↓ Dilute to 250 ml with distilled deionized water

Take a 5 ml aliquot

↓ Add 10 mg of lyxose and dilute to 10 ml with distilled deionized water

Total of 10 ml solution with internal standard

↓

Inject 10 µl into HPLC for analysis

Chart 1. Hydrolysis of cellulosic samples and preparation of solutions for liquid chromatographic analysis.

through which nitrogen was passed. The cotton was recovered by filtration, washed well with water, and then with acetone (three times) and allowed to air dry in the hood. A small Wiley mill with a 40 mesh screen was used to mill the cotton. It was then placed in an oven (105°) over-night to ensure complete water removal. This cotton powder (350 mg) was dissolved and hydrolyzed according to the procedures mentioned above (chart 1). A 5-ml aliquot was removed every 30 min from the refluxing solution and transferred to a 25-ml volumetric flask. Lyxose (10 mg) was added and distilled deionized water was added to 25 ml. An aliquot of 25 µl was injected into the liquid chromatograph for monosaccharides analysis.

C. Analyses of Cellulosic Samples by Using the Borate Buffer System

Analyses procedures for cellulosic samples were standardized as described in chart 1. Samples were put in an oven (105°) for at least 12 hr. A small Wiley mill and a 40 mesh screen were used to grind the samples into powders. The milled samples were always kept in the oven to ensure that they were oven dried. Aliquots of 350 mg of the samples were taken and dissolved in 3 ml of 77% sulfuric acid. Stirring

and squelching the samples speeded up the dissolution. The solution was then diluted to 128 ml with distilled deionized water in a 250 ml round-bottomed flask. The solutions were refluxed for 4.5 hr, filtered through a No.1 Whatman filter paper with the help of an aspirator. The insoluble part of the sample was thoroughly washed on the filter paper to ensure that it was free of monosaccharides. The filtrate was then diluted to 250 ml with distilled deionized water. An aliquot of 5 ml was taken and 10 mg of lyxose was added as an internal standard, then diluted to 10 ml. Aliquots of 10 µl were injected into the liquid chromatograph for analysis. Six commercial cellulosic samples and a commercial thermomechanical pulp prepared from hemlock wood were hydrolyzed by the procedures described above (chart 1) except that L-rhamnose was used as an internal standard.

D. Comparison of the Two Systems

Two eluant solutions were needed for the "B" system (borate buffer system) while one was required for the "A" system (acetonitrile-water system). An extra water jacket and a boiling water bath were necessary to keep the column at constant temperature and allow the monosaccharides to react with the copper dye reagent in the B system. The running time for an analysis was about 20 min for system A

but for system B was about 100 min. A differential refractometer was used for system A and an absorbance detector was used in system B. Both systems used at least one sample pump and a Hewlett Packard Model 3380A integrator.

IV. RESULTS AND DISCUSSION

A. High-Performance Liquid Chromatography

1. Acetonitrile-Water System

The acetonitrile-water system worked well for the individual sugars. However, there was poor resolution between galactose, mannose and glucose (Figure 1). The pentoses, arabinose and xylose, were well resolved. Myo-inositol proved to be a good internal standard because its retention time was longer than the other sugars. Thus it eluted after the other sugars and did not interfere with them. The instrument "K" factors were good for the acetonitrile system as shown in Figures 2-7. These provided good straight lines. The slope of the curve resulting from plotting the weight ratio of the sugar to myo-inositol versus the peak area ratio of the sugar to myo-inositol provides the instrument "K" factor. This factor is used with internal standards because the detector may not respond equally to the internal standard and the sugars. The "K" factors were: rhamnose, 0.735 (Figure 2, R^2=0.995); xylose, 0.735 (Figure 3, R^2=0.981); arabinose, 0.753 (Figure 4, R^2=0.989); galactose, 0.660 (Figure 5, R^2=0.989); mannose, 0.643 (Figure 6, R^2=0.997); glucose, 0.940 (Figure 7, R^2=0.998). These data were obtained from linear regression analyses.

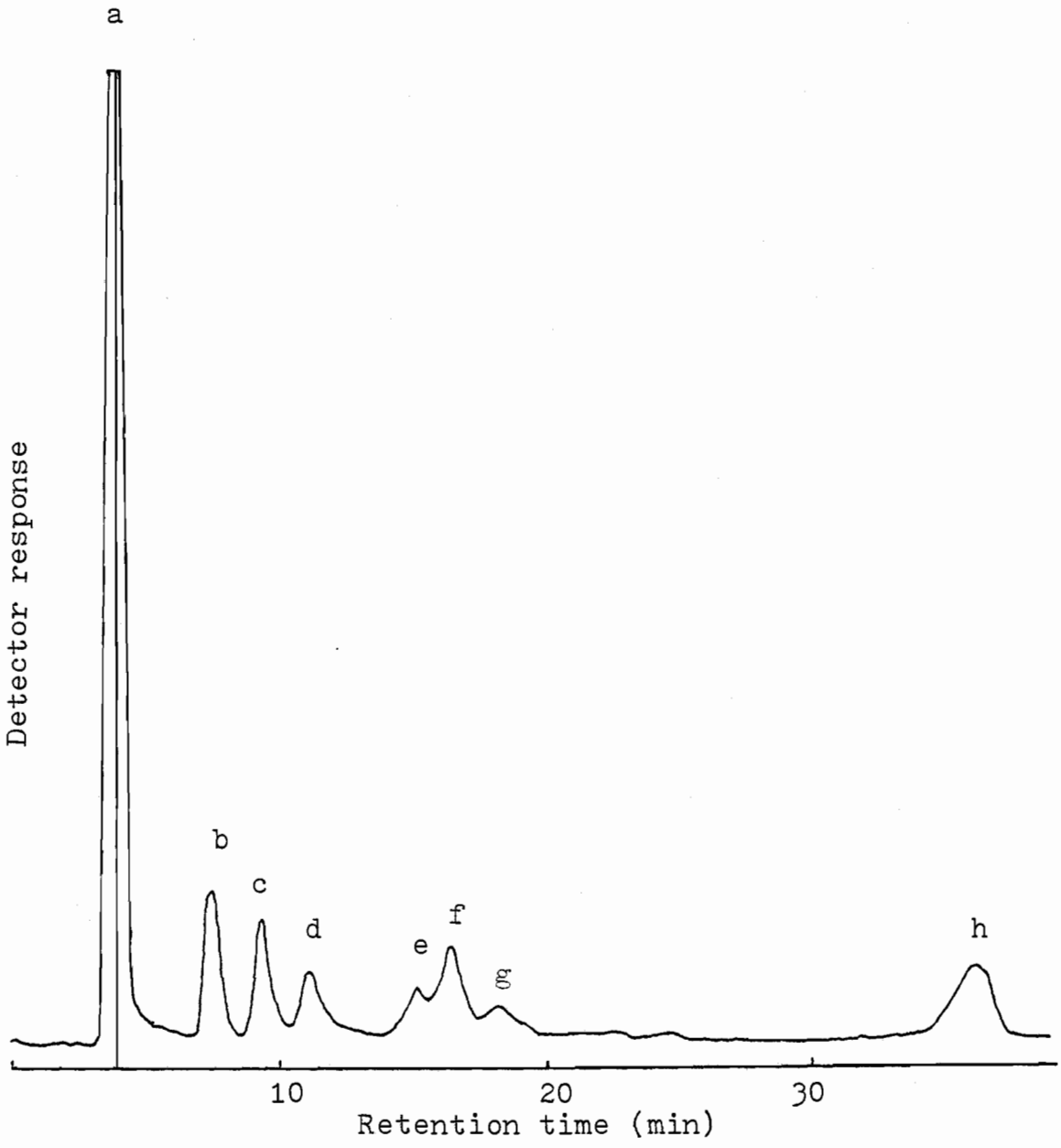


Figure 1. Liquid chromatographic spectrum of a solution of authentic monosaccharides with the acetonitrile-water system. Peak "a" is from the solvent (H_2O), "b" is from rhamnose, "c" is from xylose, "d" is from arabinose, "e" is from mannose, "f" is from glucose, "g" is from galactose, "h" is from myo-inositol. Conditions: column, Waters Associates "μ BONDAPAK/Carbohydrate"; eluant, acetonitrile-water 85:15 v/v; flow rate, 1.7 ml/min; detector, differential refractometer.

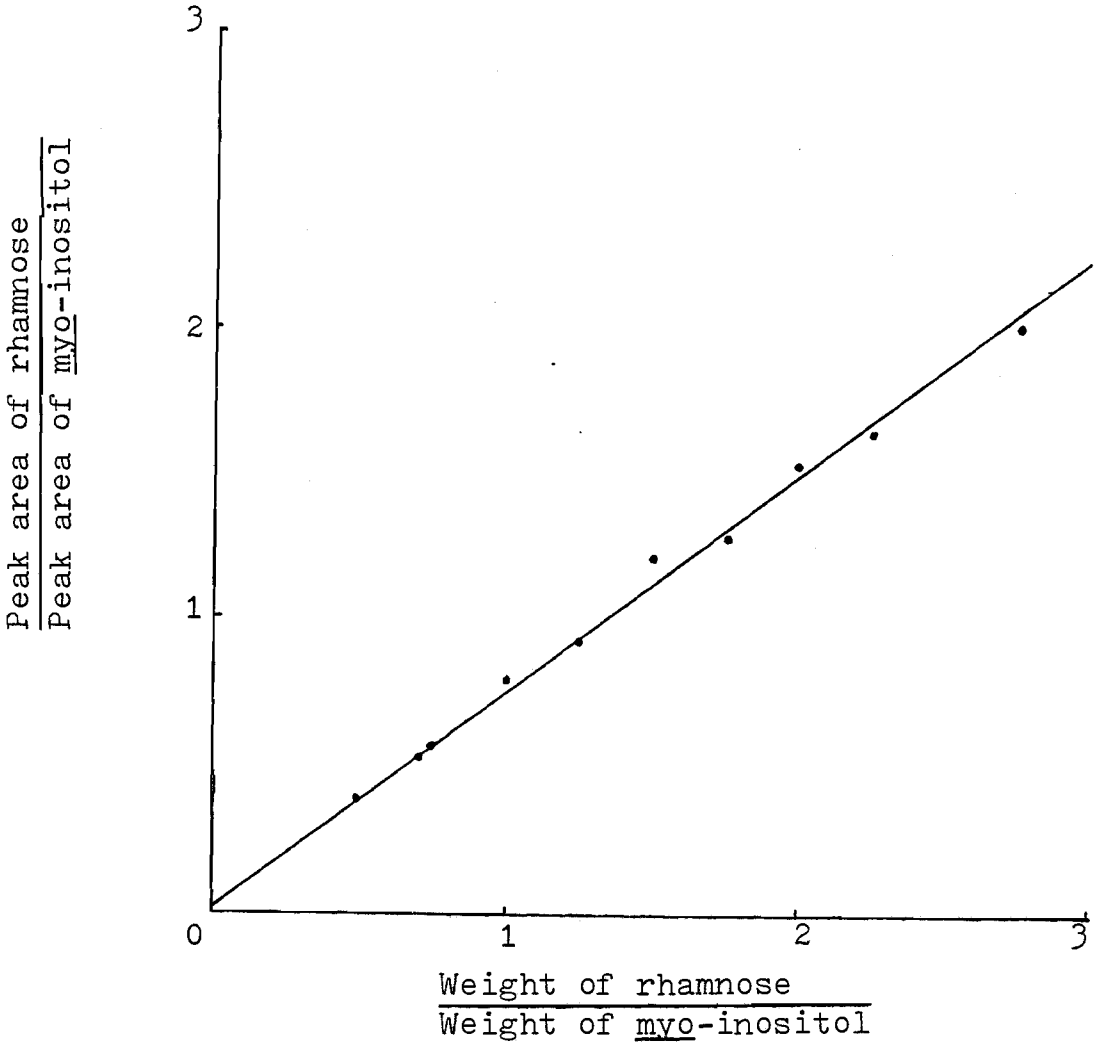


Figure 2. Recovery of authentic rhamnose to determine an "Instrument K Factor" for the acetonitrile-water system.

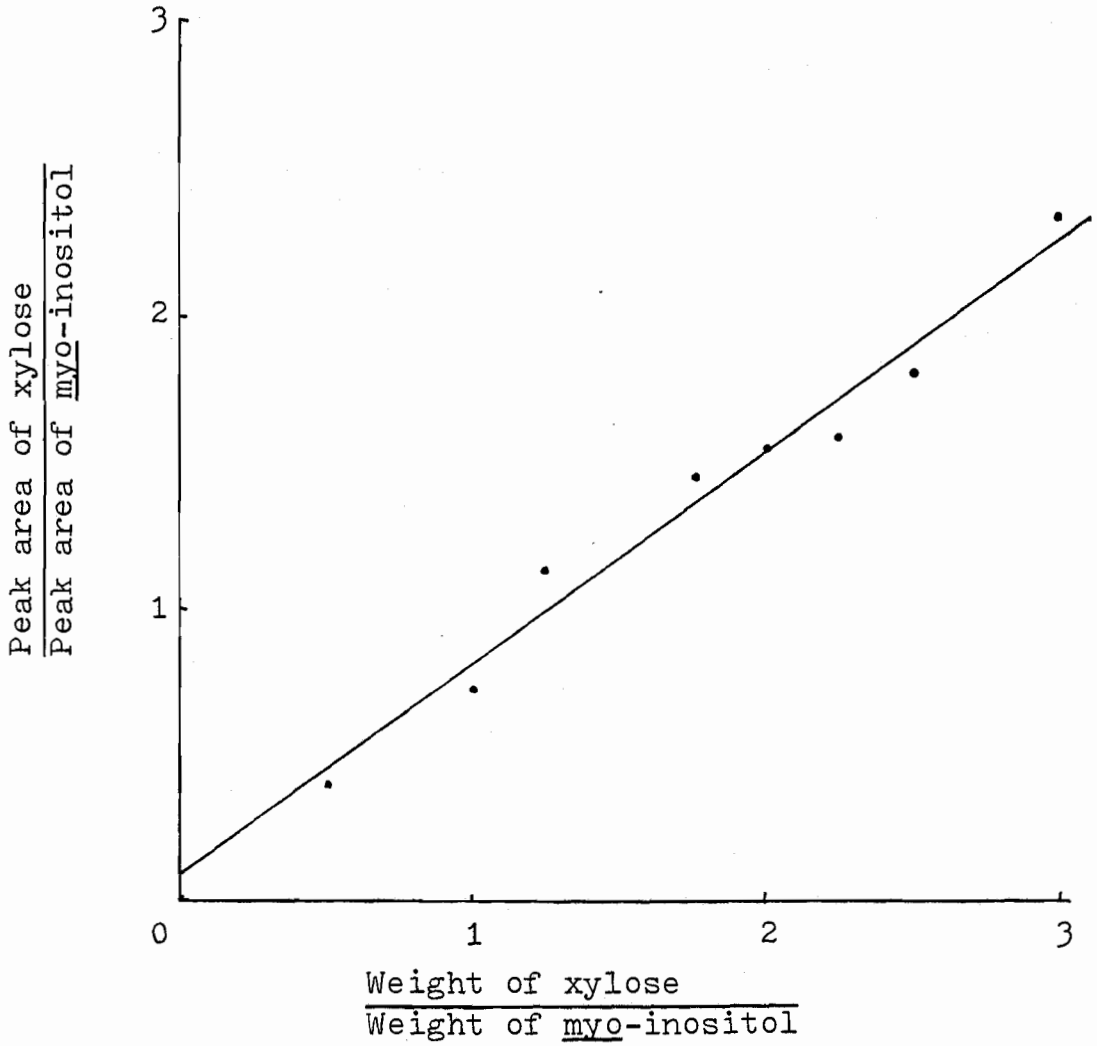


Figure 3. Recovery of authentic xylose to determine an "Instrument K Factor" for the acetonitrile-water system.

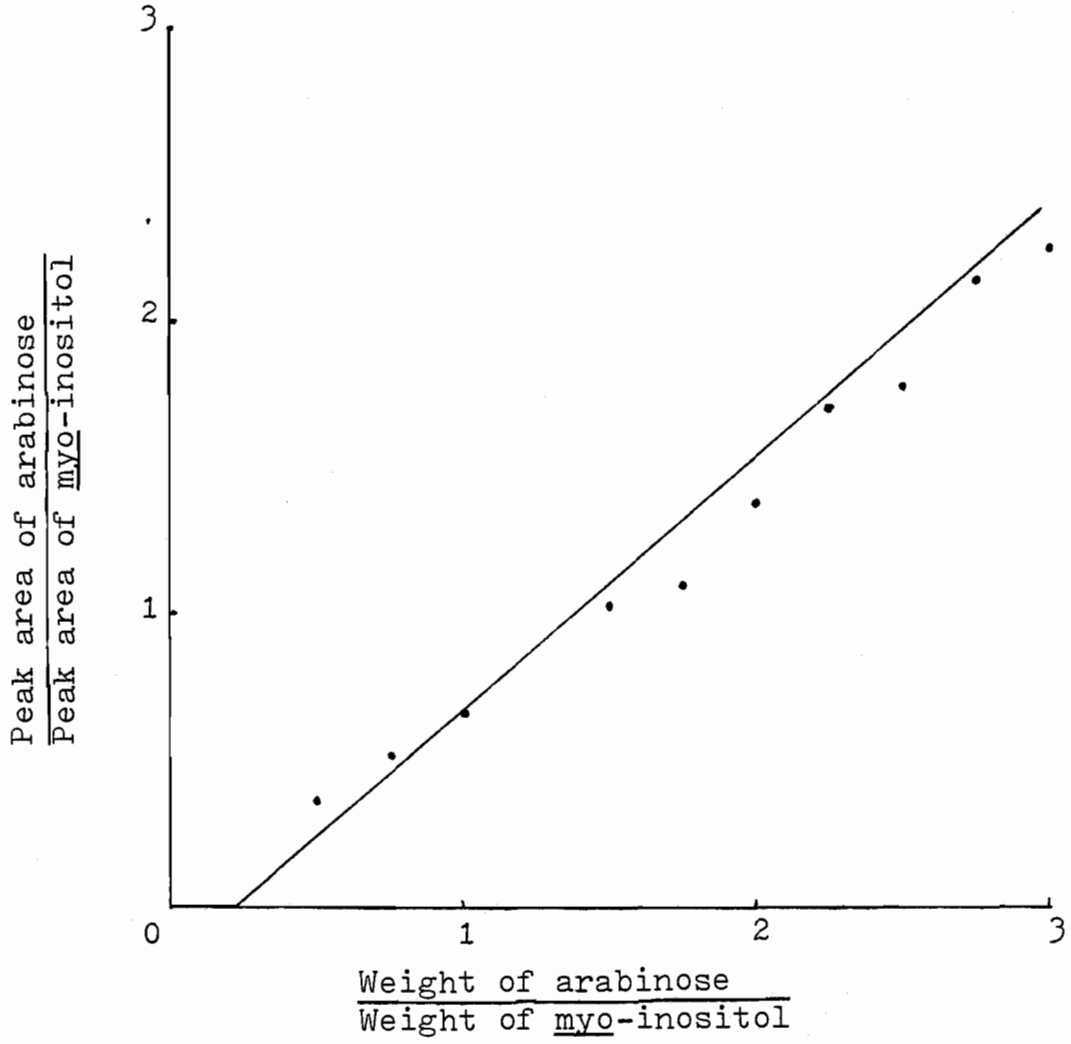


Figure 4. Recovery of authentic arabinose to determine an "Instrument K Factor" for the acetonitrile-water system.

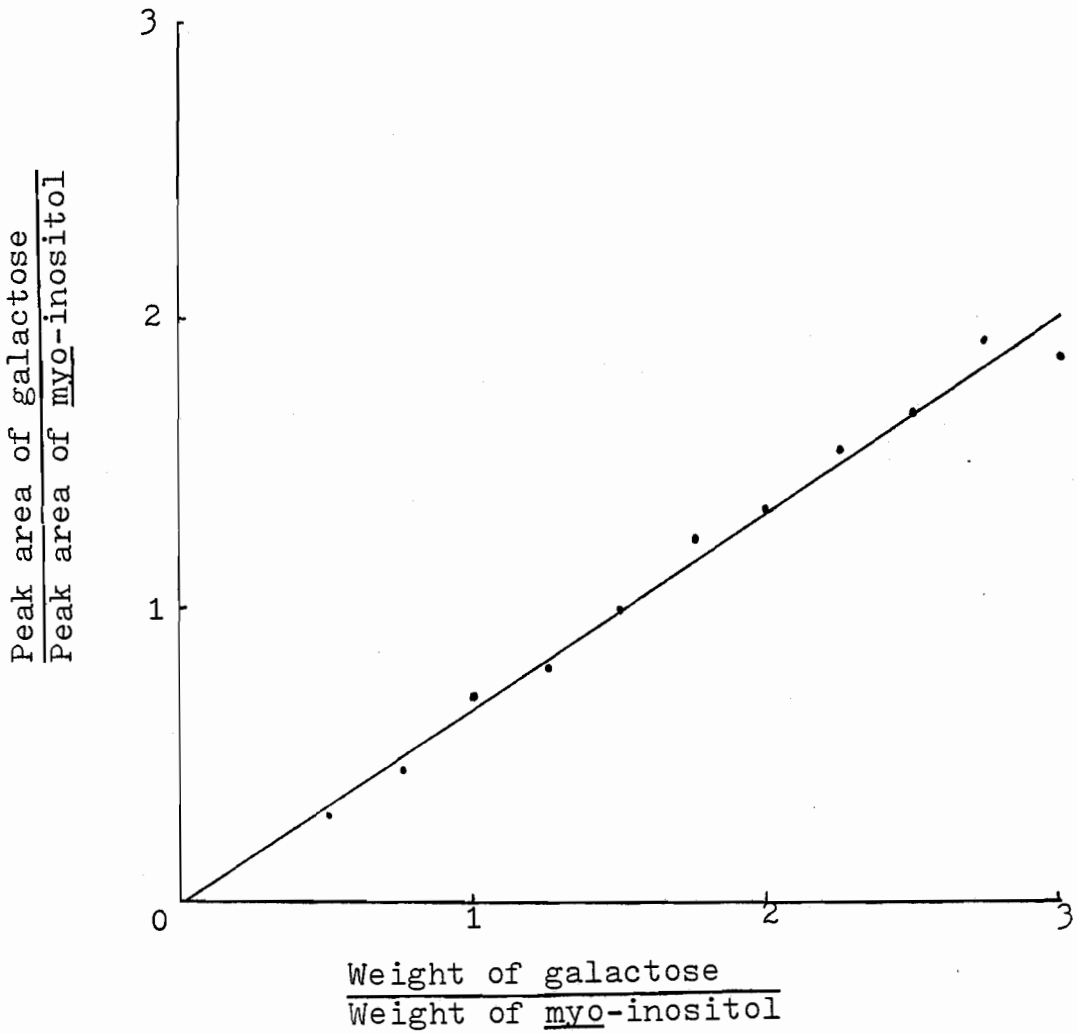


Figure 5. Recovery of authentic galactose to determine an "Instrument K Factor" for the acetonitrile-water system.

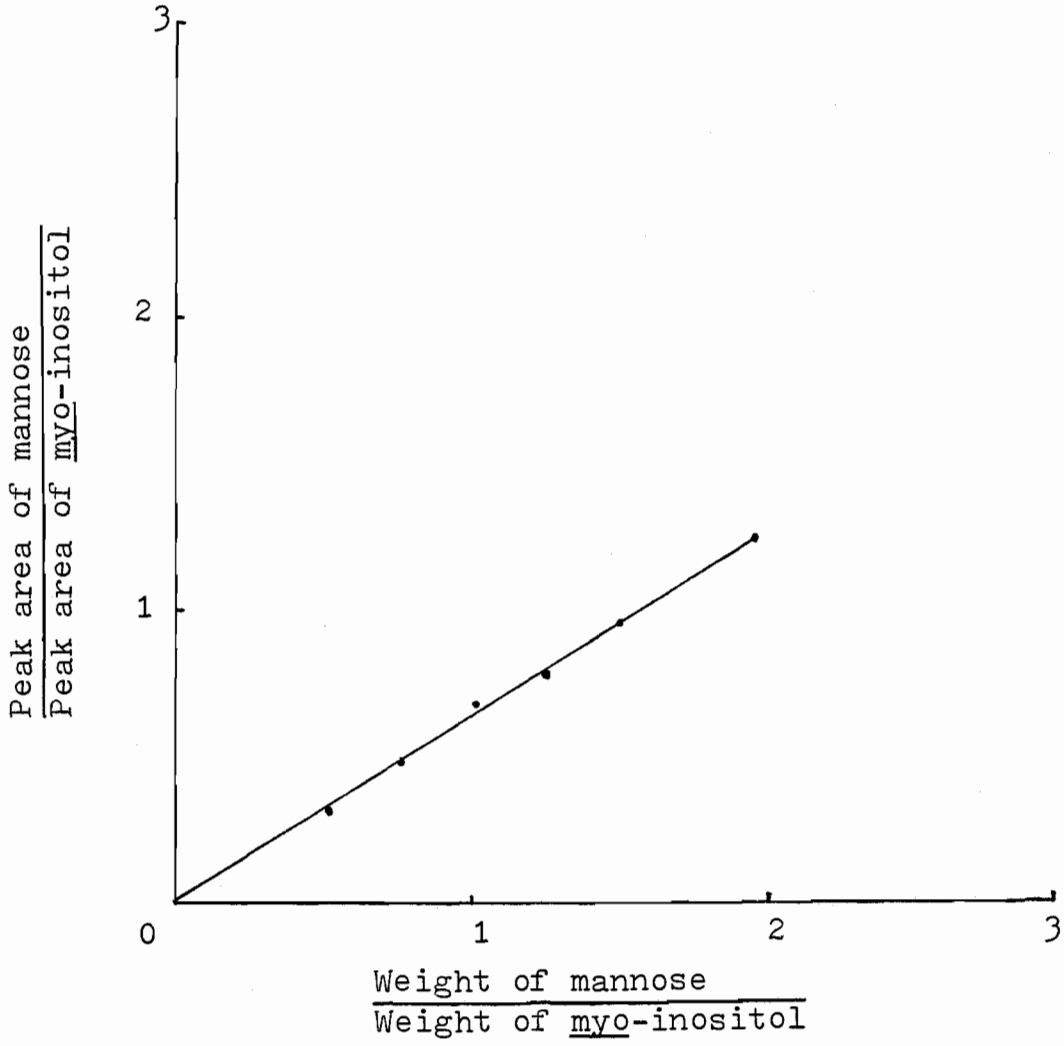


Figure 6. Recovery of authentic mannose to determine an "Instrument K Factor" for the acetonitrile-water system.

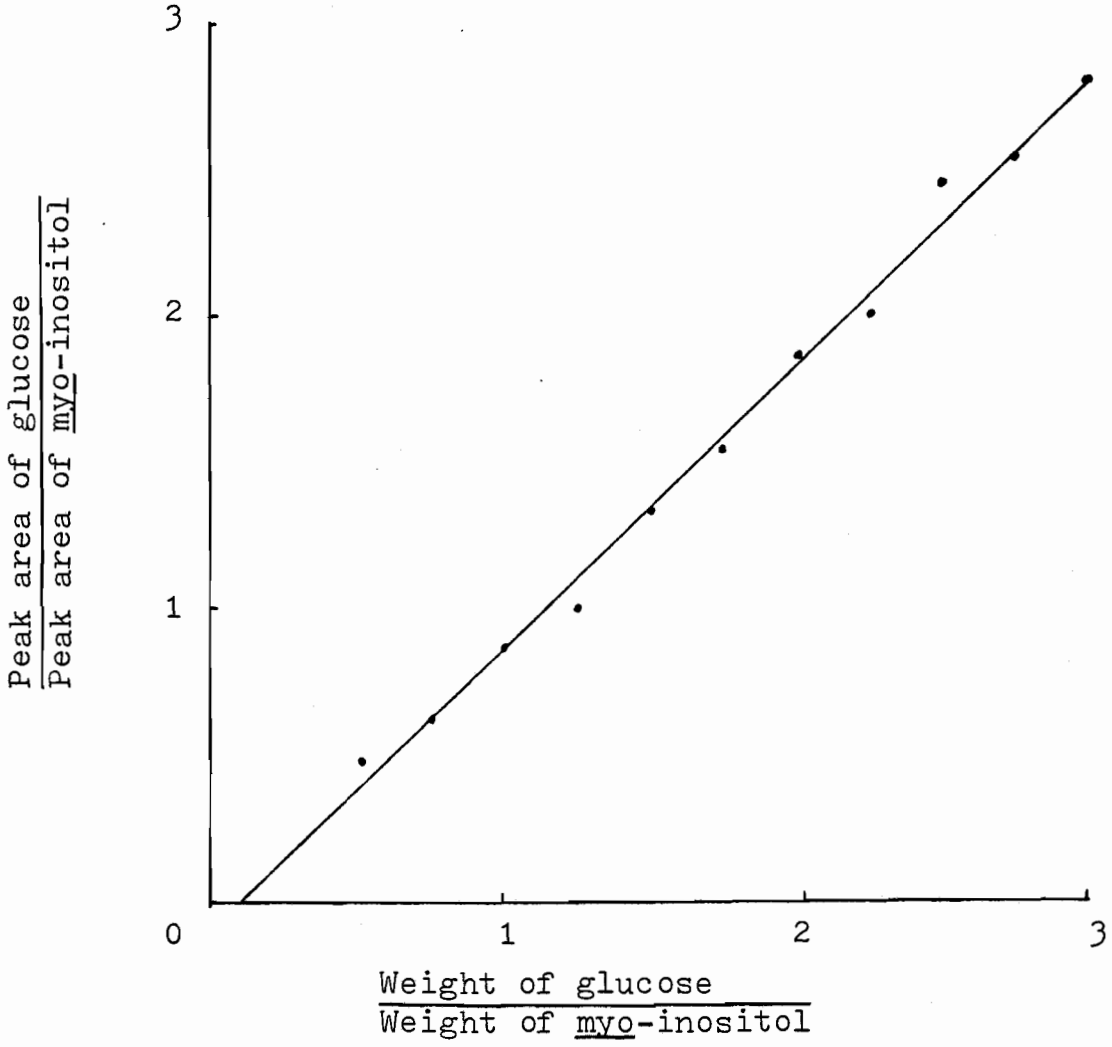


Figure 7. Recovery of authentic glucose to determine an "Instrument K Factor" for the acetonitrile-water system.

2. Borate Buffer System

a. Borate Buffer Preparation The concentration of the borate buffer was reported by Sinner, Simatupang and Dietrichs (34) to be colsely related to separation time (34). The buffer with the higher concentration shortened the retention time of glucose from 225 min (0.31 M) to 105 min (0.49 M). There are two factors to be considered in determining the most suitable concentration of buffer to use, the time, and the separation. The buffer of the lowest concentration got the best separation but with broadened peaks and longer retention time. Having tried to get the best separation and the least time consumed, 0.13 M in potassium borate ($K_2B_4O_7$) was found to be the best compromise with 0.2 ml/min flow rate. Also the lower buffer molarity suggested more accurate measurements of the minor components in a sugar mixture (34). Due to the folw rate limits found with this anion-exchange column and the fact that faster flow rates didn't mean shorter retention time, 0.13 M potassium borate run at 0.2 ml/min with a retention time of 100 min for glucose was satisfactory. A more concentrated (10 times as concentrated as the eluant buffer used in this laboratory) was suggested for flushing the column when contamination occurred (10). It was found effective to do so, but the cleaning of the column usually did not last long. Repacking the column with regeneration of the resin was required in most cases.

b. Copper Dye Reagent Preparation Monosaccharides are colorless when they elute from the column. To detect their presence, a differential refractometer detector or an absorbance detector are often used. However, it was found that the differential refractometer detector did not detect the presence of the monosaccharides in this borate buffer solvent system, although it was efficiently used in the acetonitrile-water system (28). The only purpose in adding the copper dye reagent was to react with the monosaccharides separated by the column and make them detectable. The mechanism will be fully discussed in "Section f". Solution A and solution B can stand on the shelf for weeks without being changed by time. However, once they are mixed the solution does not last too long. The absorbance of light at 546 nm wavelength increased about 0.030 units on the monitor scale each day if the offset absorbance was about 0.400 when freshly made. The color of this copper dye reagent will eventually turn purple. The detection capacity is thus decreased with increased storage time.

c. Column Preparation

The column, as always, was found to be the heart of the chromatographic analysis. The resin used was Durrum, Chromex DA-X4-11 from Dionex Corp., Sunnyvale, CA.. Generally speaking

this resin provided a satisfactory separation. It is a spherical resin copolymer of divinylbenzene cross-linked with quaternary amine functional groups. It is supplied fully hydrated in the chloride form and typically contains 50% water, which is dependent on the degree of cross-linking. For our use, it was transferred from the chloride form to the borate form by washing with the borate buffer solution several times. After the column was filled with resin slurry, it was very critical to pack it with the flow rate that was goint to be used in the actual separation, 0.2 ml/min in this case. Pumping buffer through the column for at least 2 hr was necessary for conditioning the resin bed before injecting a sample. A prefilter was used in the line ahead of the column to prevent any contamination that might exist in the samples injected. A vibrator was found to be very useful while packing the column to shake out trapped air from the slurry. The vibrator also helped distribute the resin bed evenly. It was also found to be important that the column be full of resin. Unless the column was full of resin, channels developed which allowed the eluant buffer to stream through the column without passing through the resin bed. This interfered with sugar separation and caused shoulders on the backsides of the peaks. The back pressure of the column ranged between 0 and 500 psi, if the column was packed correctly. However, after a period of running time, the pressure often increased because of

contamination. Under these circumstances, the flow direction of the column was changed by turning the column end-for-end. Pumping through concentrated borate buffer (10 times as concentrated as that used for sugar separations) to flush away the contaminates was also sometimes helpful in solving the problem. However, the effect usually did not last very long. It was found that the best way to keep the machine running was to : 1) keep the eluant solvent flowing all the time at the same flow rate; 2) keep the injected sample solution clean. It must be filtered through a 0.45 μm filter; 3) clean the prefilter periodically; 4) never shut down the hot water bath. Keeping the eluant solvent flowing also had the advantage that it avoided the risk of crystallization of the buffer in the column and tubings. It was found that there were always difficulties in getting back the original conditions if the instrument had been shut down. The resin bed might have become distorted by different flow rates or lowering of the temperature.

d. Equipment

Figure 8 shows a schematic diagram of the apparatus used for carbohydrate analyses. Initially both pumps A and B were Waters Associates, Model 6000A duel piston high-performance liquid chromatographic solvent delivery systems. Later pump

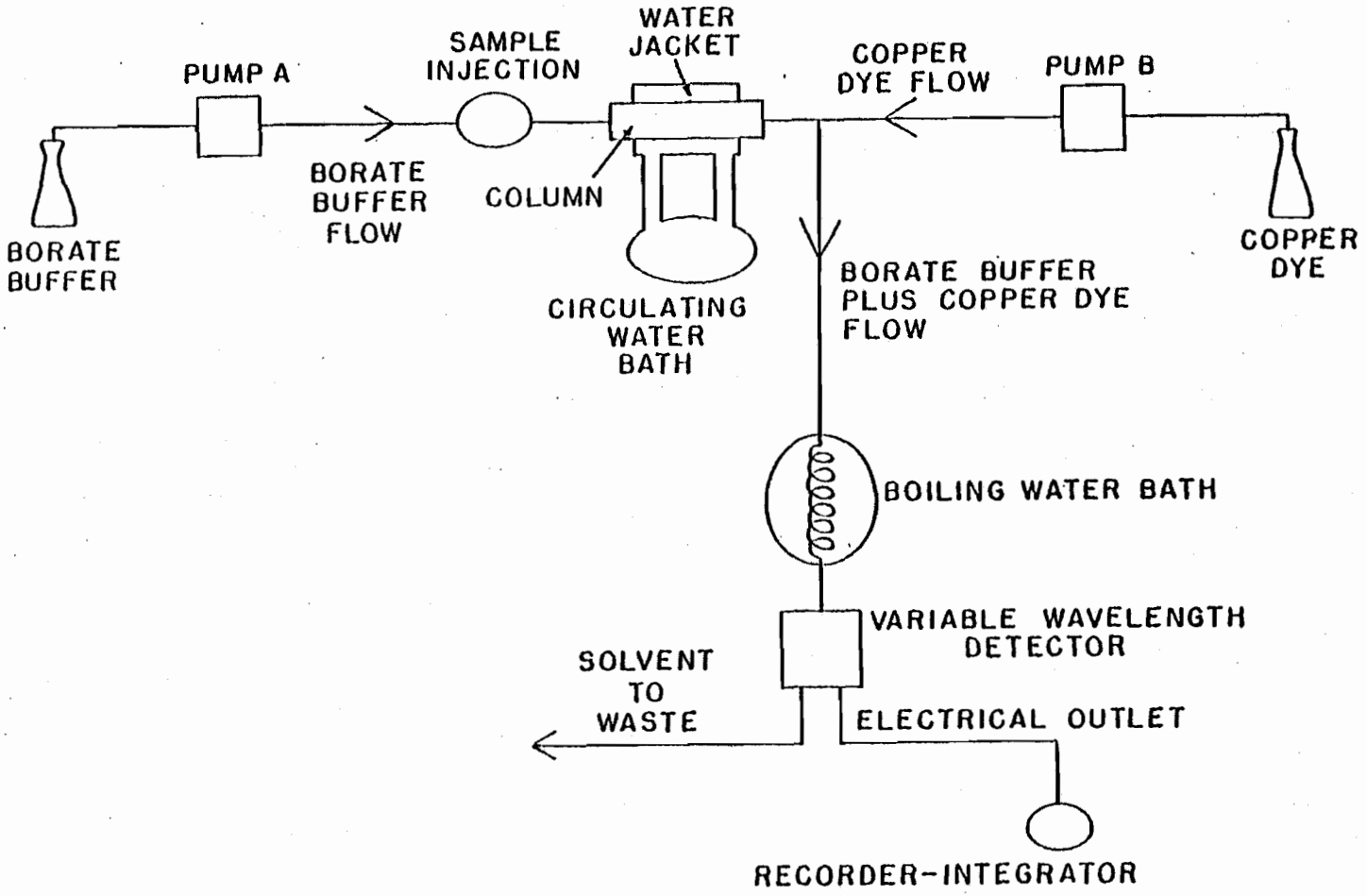


Figure 8. Schematic diagram of the apparatus used for carbohydrate analyses.

B was replaced with a small, single piston pump from Eldex Inc. which worked well.

The temperature of the column was found to be imporatant. It had to be maintained at about 55°. The temperature should not be too high because the monosaccharides are sensitive to high temperature. A lowered temperature tended to build backpressure which ultimately resulted in a plugged column. The column was kept at 57.5° by pumping water through a water jacket from a Haake circulating water bath (Figure 8).

The eluant from the column was mixed with the copper dye in a T joint (Figure 8). Friberg, Barnes, and Meyer (10) suggested a mixing chamber with a small star magnetic stirring bar to mix the eluant and the dye but with the low flow rates used in the present work this was found to be unnecessary.

A 15 m length of Teflon tubing (0.8 mm internal diameter) carried the mixed column eluant and dye through the boiling water bath (Figure 8). The boiling water bath was used to heat the sugars and the dye to bring about the reaction which resulted in color formation. The time the sugar and the dye spent in the boiling water bath was calculated as follows:

$$\pi r^2 \times \text{length} = \text{volume of Teflon tubing}$$

$$3.1416 \times \left(\frac{0.8 \text{ mm}}{2}\right)^2 \times 1500 \text{ mm} = 7.54 \text{ cm}^3$$

The volume pumped is a total of 0.7 ml/min (0.2 ml/min borate buffer + 0.5 ml/min of dye). Therefore, the time required to

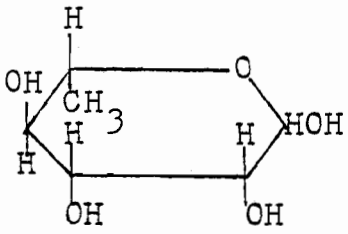
pump the solution through the Teflon tubing in the boiling water bath is then $\frac{7.54\ cm^3}{0.7\ ml/min}$ = 10.8 min.

It had been determined that the heating time required for maximum color development between the sugars and the dye was at least 15 min. Therefore, a time in the boiling water bath of 10.8 min was insufficient for complete reaction between the sugar and the dye. Since there is an incomplete reaction it is critical that all conditions of the analysis be maintained constant. For example, if the length of tubing in the boiling water bath is changed, then the amount of color developed will change and the absorption results will be erroneous.

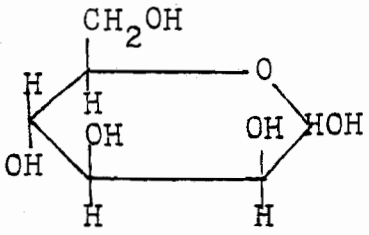
The wavelength of maximum absorbance of the color developed between the monosaccharides and the dye was found to be 560 nm the same as that reported by Sinner, Simatupang and Dietrichs (34). However, the closest filter which could be used with the Waters Associates instrument was of 546 nm.

e. Separation of Monosaccharides

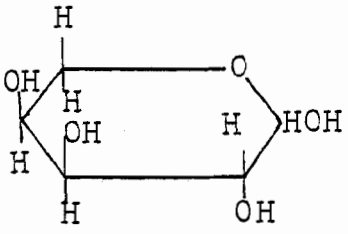
Six monosaccharides are found in acid hydrolyzates of wood:



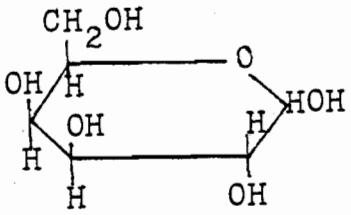
L-rhamnose



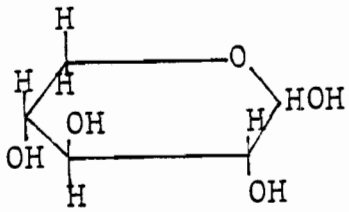
D-mannose



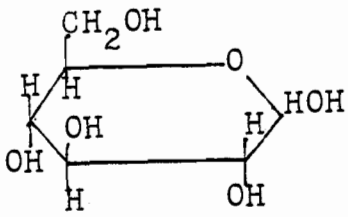
L-arabinose



D-galactose



D-xylose



D-glucose

Some are different by molecular weight but all are different by chemical configuration. As mentioned in the historical review, borate ions react with monosaccharides to form complexes and the ease of complex formation and the stability of the complexes are undoubtly due to the stereochemistry. It has been established (9) that the greater the number of hydroxyl groups in axial positions, the less stable will be the formed complex. It has also been found that D-glucose, which possesses all of its hydroxyl groups in equatorial positions moves fastest in electrophoretic analysis (09). These findings indicate that the borate buffer complexes with trans-hydroxyl groups easier than with cis-hydroxyl groups and once formed a more stable complex results.

There are two other factors which influence the separation pH and the concentration of the borate ions (9,34). It has been reported (9) that higher pH's increase the concentrations of the ionic species and thus increased the electrophoretic mobility of the monosaccharides. At pH 8, the mobility of the carbohydrates was found to be proportional to the borate content of the buffer, suggesting that, under these conditions,

some of the carbohydrates in solution were uncomplexed. As for the column, the resin was exchanged to the borate form by washing with concentrated borate buffer.

Therefore, it is then suggested that the mechanism of separation of the monosaccharides is as follows. The monosaccharides complex with the borate ions right after they are injected into the liquid chromatograph and mix with the borate buffer. The stability of these complexes varies according to their specific configurations, for example, D-glucose can form the most stable complex while L-rhamnose forms the least stable complex according to their stereochemistry. However, in this dilute borate buffer solution, many of them are uncomplexed (9). However, after they enter the column, they have more chances to complex with the borate ions that are attached to the resin. The sugar that forms the most stable complex will then stay longer on the resin, and vice versa. Increasing the concentration of the eluant borate buffer would shorten the retention time (9) because the monosaccharides would tend to complex with the borate ions in the eluant and would have less chance to complex with the borate ions on the resin. Due to this equilibrium of a monosaccharide complexing with the borate ion on the resin, a mixture of authentic monosaccharides can be separated (Figure 9).

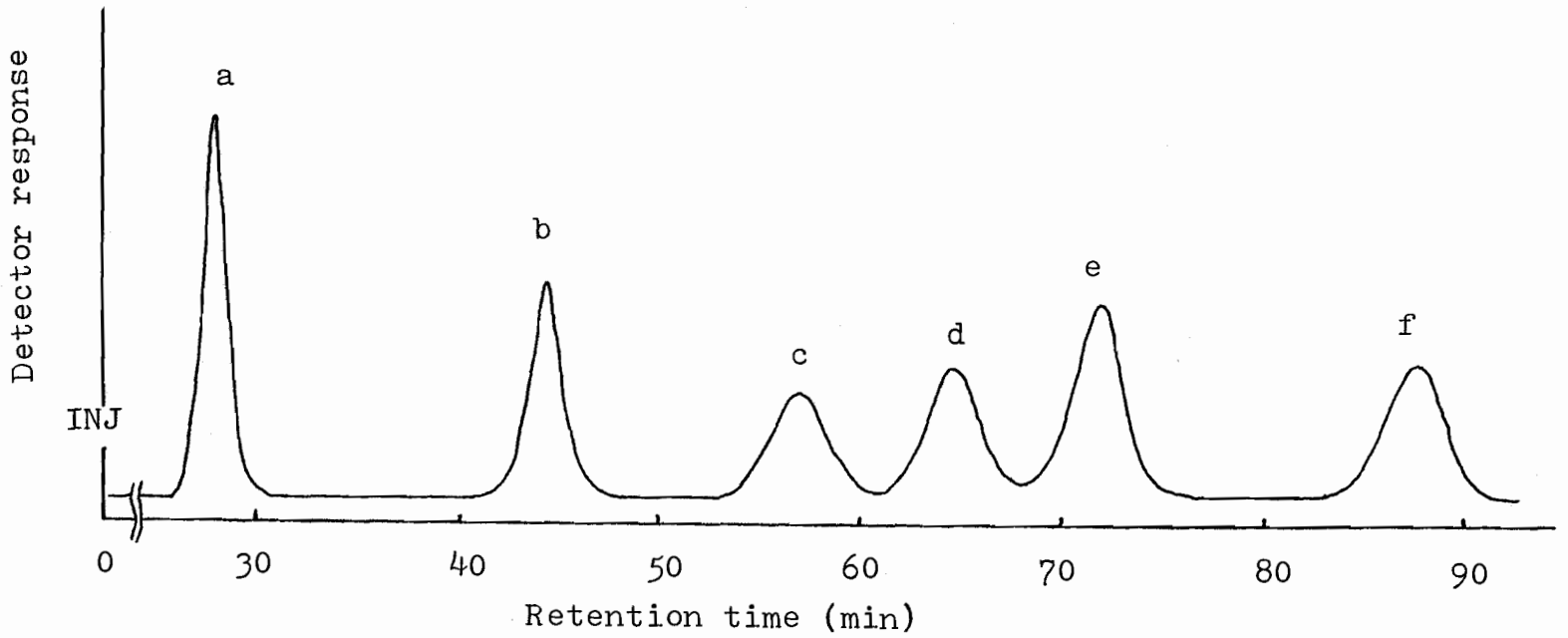
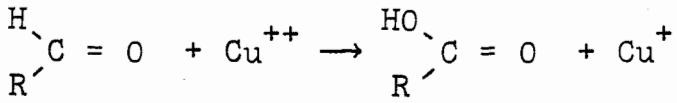


Figure 9. Liquid chromatographic spectrum of a solution of authentic monosaccharides with the borate buffer system. Peak "a" is from rhamnose, "b" is from mannose, "c" is from arabinose, "d" is from galactose, "e" is from xylose, "f" is from glucose. Conditions: column, Chromex DA-X4-11; eluant, 0.13 M potassium borate buffer; flow rate, 0.2 ml/min; detector, copper dye and absorbance monitor set at 546 nm.

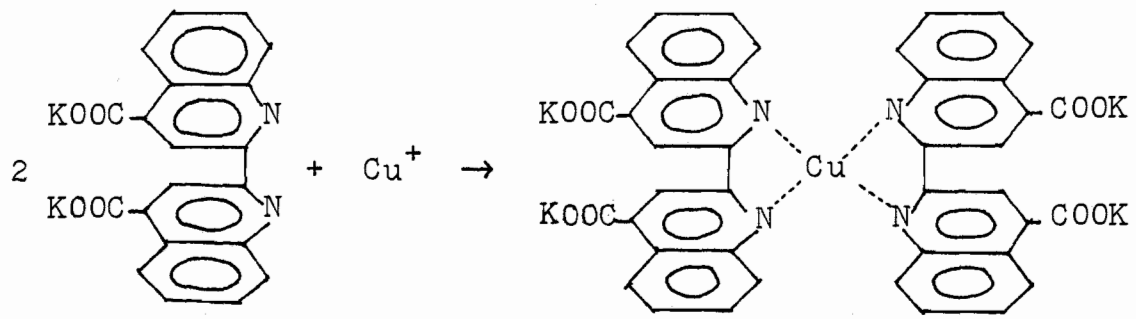
f. Maximum Absorbance and Mechanism of Detection

The monosaccharides are colorless when they elute from the column. A copper dye reagent was added and boiled to make the presence of the monosaccharides detectable by an absorbance monitor.

It is well known that sugars are reducing compounds. The cupric ion Cu^{++} can be reduced to the cuprous ion Cu^{+} by reaction with sugars according to the equation:



It is actually the cuprous ion Cu^{+} reduced by the sugars that we are detecting to determine how much sugar is in the solution by forming a lavender-colored chelate complex with 2,2'-bicinchoninic acid (dipotassium salt) as shown:



The bicinchoninic acid thus forms a lavender-colored bis complex with cuprous ions. The maximum absorbance in the visible region is at 560 nm. The complex is reported to be stable at pH 4-12 and obeys Beer's law to 100 mg/liter of copper (11).

The reagent is water soluble and affected by few metal interferences. This method was first reported in 1961 by the Russian chemists Gèrshuns, Verezubova and Tolstykh (11). And was originally developed to detect trace copper ions in metal alloys (12,26,27). Gindler applied this method to detect the glucose content in blood serum in 1970 (14). Mopper and Gindler developed it as a new noncorrosive dye reagent for automatic sugar chromatography in 1973 (25). The system was further modified by Sinner and Puls in 1978 (35). Since the complex that the detector was monitoring was the same no matter what the monosaccharide was, glucose was taken as "representative" in this experiment. The color was scanned from 700 nm to 450 nm (Figure 10). The maximum absorbance was found to occur at 560 nm, the same as reported previously (14). The absorbance at 546 nm was also measured because this was the wavelength measured by the absorbance detector used. It was used because it was the closest one available and the absorbance was only 0.030 reading divisions below the maximum absorbance.

g. Effect of Heating Time on the Copper Dye Reagent Alone

The procedures which were used to prepare the standard solution were also used to prepare the dye reagent alone except that the sugars were omitted. The results are shown in Table 1 and Figure 11. A non-linear relationship between absorbance and boiling time is clearly shown. This increased absorbance

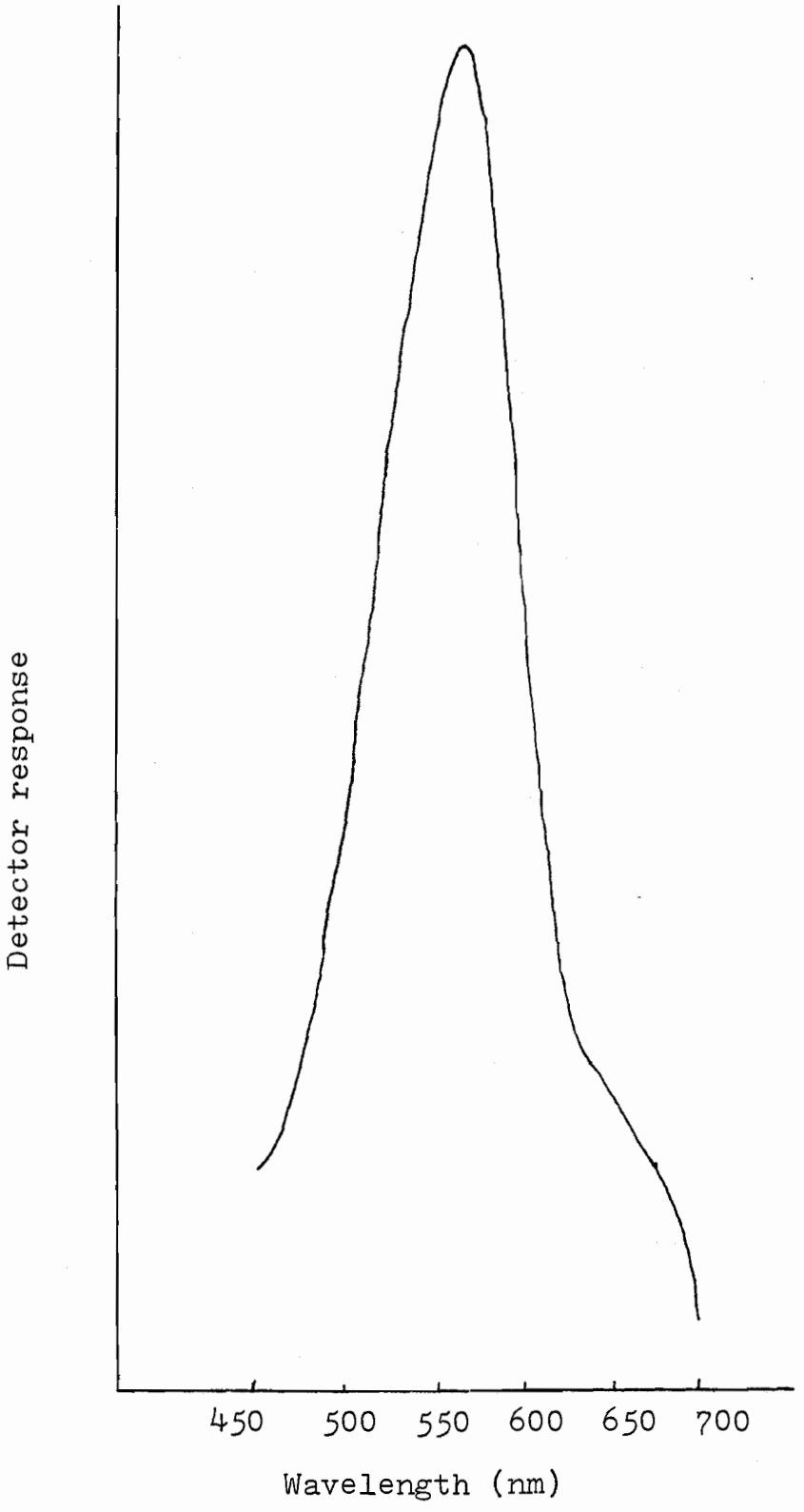


Figure 10. Absorbance spectrum of the complex formed by reaction of cuprous ion and dipotassium 2,2'-bicinchoninate.

Table 1. Effect of heating time on copper dye reagent itself

Heating time (min)	Reading of monitor
1	0.022
2	0.027
3	0.039
4	0.046
5	0.060
10	0.085
15	0.098
20	0.120
25	0.137
30	0.141

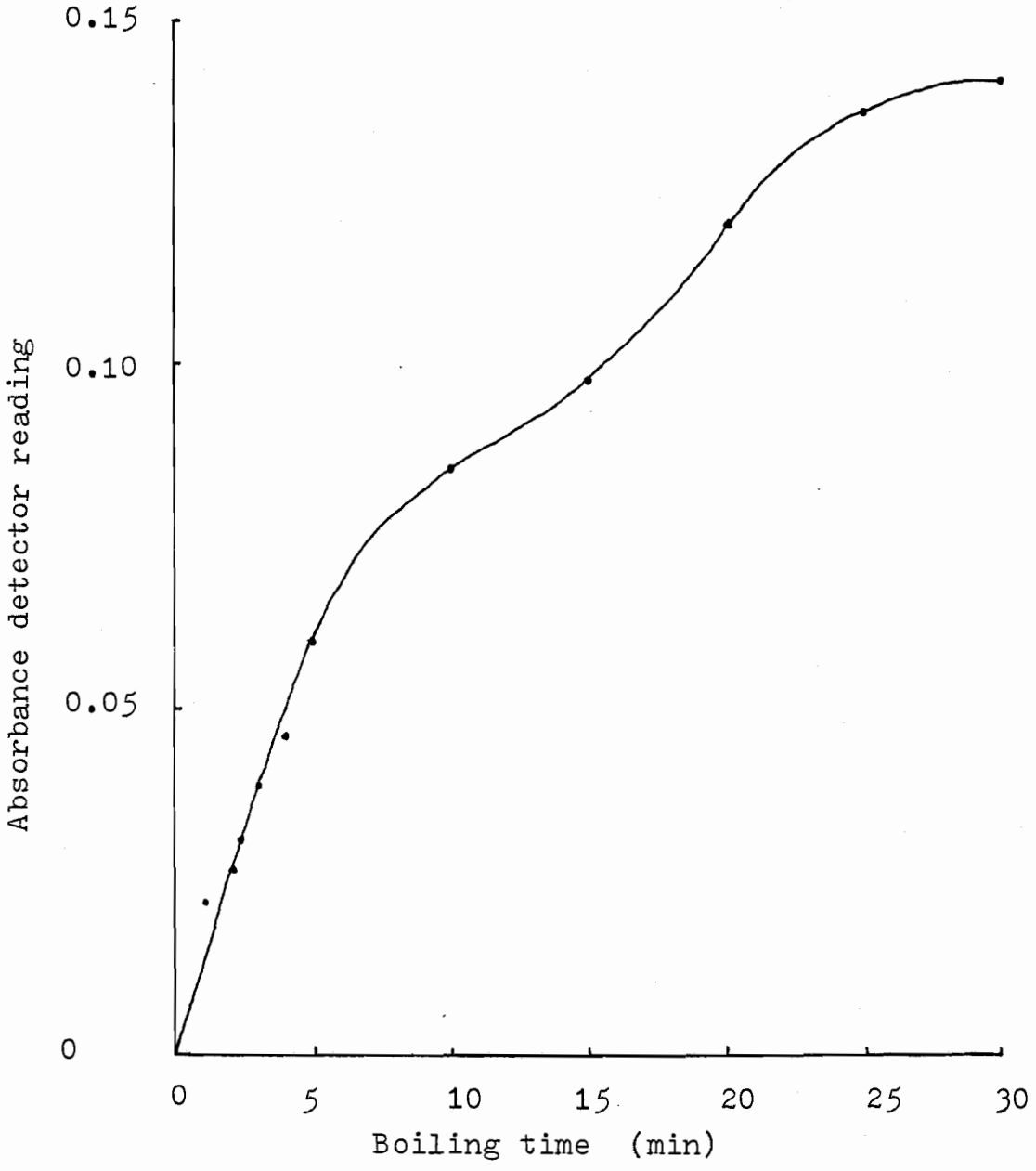


Figure 11. Effect of heating time on copper dye reagent alone.

due to boiling the copper dye reagent alone must be subtracted from the total absorbance to derive the real absorbance caused by the monosaccharide.

h. Effect of Heating Time on the Reaction of the Copper Dye and the Monosaccharides

Figures 12 and 13 show the effect of increased heating time on the reaction of the copper dye reagent with glucose and lyxose. It appears that in both cases the reaction of the monosaccharides and the copper dye reagent was not complete even after 30 min. This was not just because of the reason mentioned in the last section that the copper dye reagent itself kept increasing in absorbance with increased heating time. Even after substracting this effect, the monosaccharides seemed to continue to react with the copper dye reagent and gave more intensive absorbance with increased heating time. Because of this, one can not say how long to heat the copper dye and the sugars to bring about complete reaction and hence a stable absorbance. Thus the most favorable condition of a stable absorbance was not possible with this detection method. It was also observed from the graphs that the rate of color formation between the copper dye and glucose and the rate of color formation between the copper dye and the internal standard lyxose were different. Because the color continues to change with heating and because the rate of color development with the internal standard is different than with the other monosaccha-

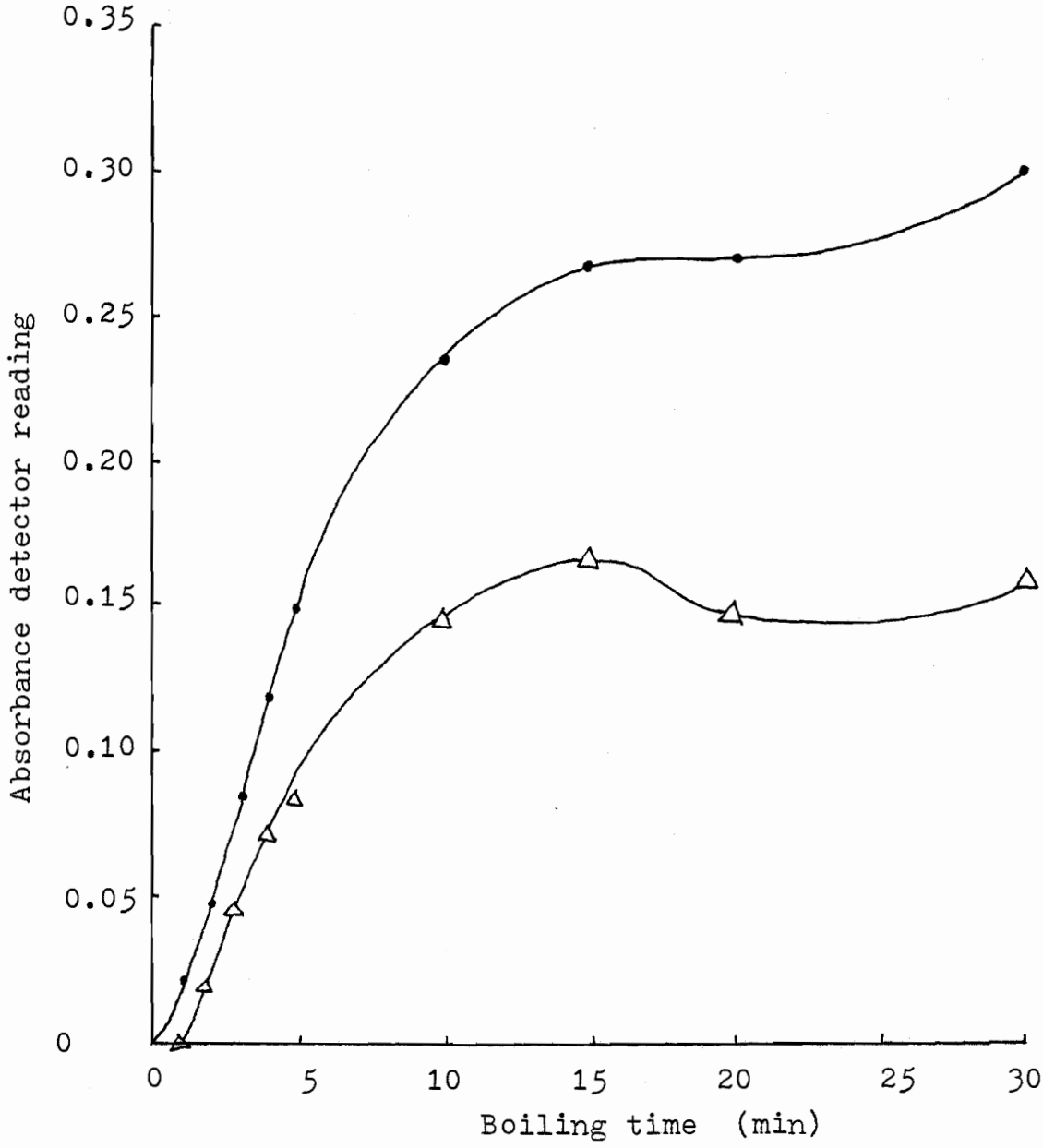


Figure 12. Effect of heating time on the reaction of the copper dye and D-glucose.
●-●-●-● includes the effect due to the dye
△-△-△-△ subtracts the effect due to the dye

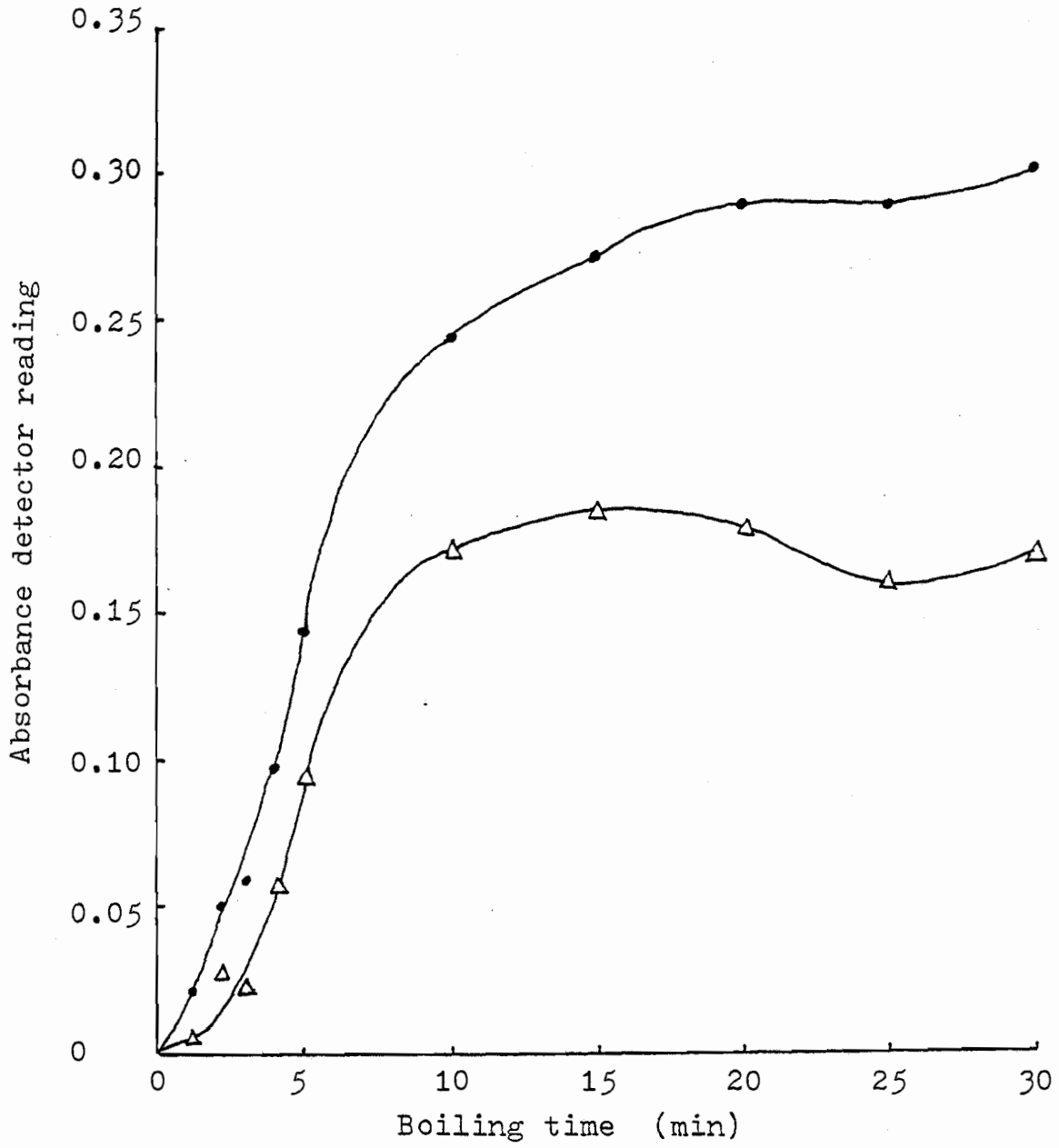


Figure 13. Effect of heating time on the reaction of the copper dye reagent and D-lyxose.
●●●●●● includes the effect due to the dye
△△△△△ subtracts the effect due to the dye

rides, it becomes absolutely necessary to standardize the heating time for every analysis. This means that the internal diameter and length of Teflon tubing in the boiling water bath must always be constant (Table 2 and Table 3).

i. Obedience of Beer's Law

Figure 14 and the regression data obtained show that Beer's law was obeyed up to 65 mg in 10 ml of solution. According to our calculations and the experimental evidence, the content of glucose never exceeded 10 mg in 10 ml of solution when prepared by the standard hydrolysis procedure. Therefore, Beer's law is obeyed for our reaction conditions. The mathematical expression which fits the best straight line is :

$$Y = 0.018 + 0.016\ X \quad \text{and } R^2 = 0.971$$

j. Internal Standards and Standard Curves

By plotting the weight ratio of monosaccharide to the internal standard versus the area ratio calculated from the chromatograms, an instrument K factor for glucose was determined. It was found that the curve which best described the relationship between the internal standard, lyxose and increasing concentrations of glucose was the quadratic equation :

$$Y = -0.049257 + 1.42226\ X - 0.233502\ X^2$$

$$\text{and } R^2 = 0.9948$$

where Y = area ratio (glucose to internal standard)
dependent variable

Table 2. Effect of heating time on the reaction of the copper dye reagent and D-glucose

Heating time (min)	Reading A[a]	Reading B[b]	Reading C[c]
1	0.020	0.022	0.000
2	0.048	0.049	0.022
3	0.085	0.086	0.047
4	0.119	0.121	0.075
5	0.150	0.145	0.085
10	0.235	0.232	0.147
15	0.267	0.267	0.169
20	0.272	0.270	0.150
30	0.300	0.301	0.160

[a] Control sample solution with D-glucose, not heated , used as reference.

[b] Control sample solution without D-glucose, not heated, used as reference.

[c] Calculated value by substracting the readings in Table 1 from reading B in this table. Reading C is thus the real effect of heating on the reaction of the copper dye reagent and D-glucose only.

Table 3. Effect of heating time on the reaction of the copper dye reagent and D-lyxose

Heating time (min)	Reading A[a]	Reading B[b]	Reading C[c]
1	0.022	0.027	0.006
2	0.051	0.054	0.028
3	0.059	0.064	0.023
4	0.098	0.105	0.058
5	0.145	0.150	0.095
10	0.245	0.249	0.172
15	0.271	0.280	0.185
20	0.290	0.295	0.178
25	0.290	0.294	0.162
30	0.301	0.308	0.170

[a] Control sample solution with D-lyxose, not heated, used as reference.

[b] Control sample solution without D-glucose, not heated, used as reference.

[c] Actual reading by using control sample solutions without D-lyxose but heated the same time as the reference respectively. Reading C is thus the real effect of heating time on the reaction of the copper dye reagent and D-lyxose only.

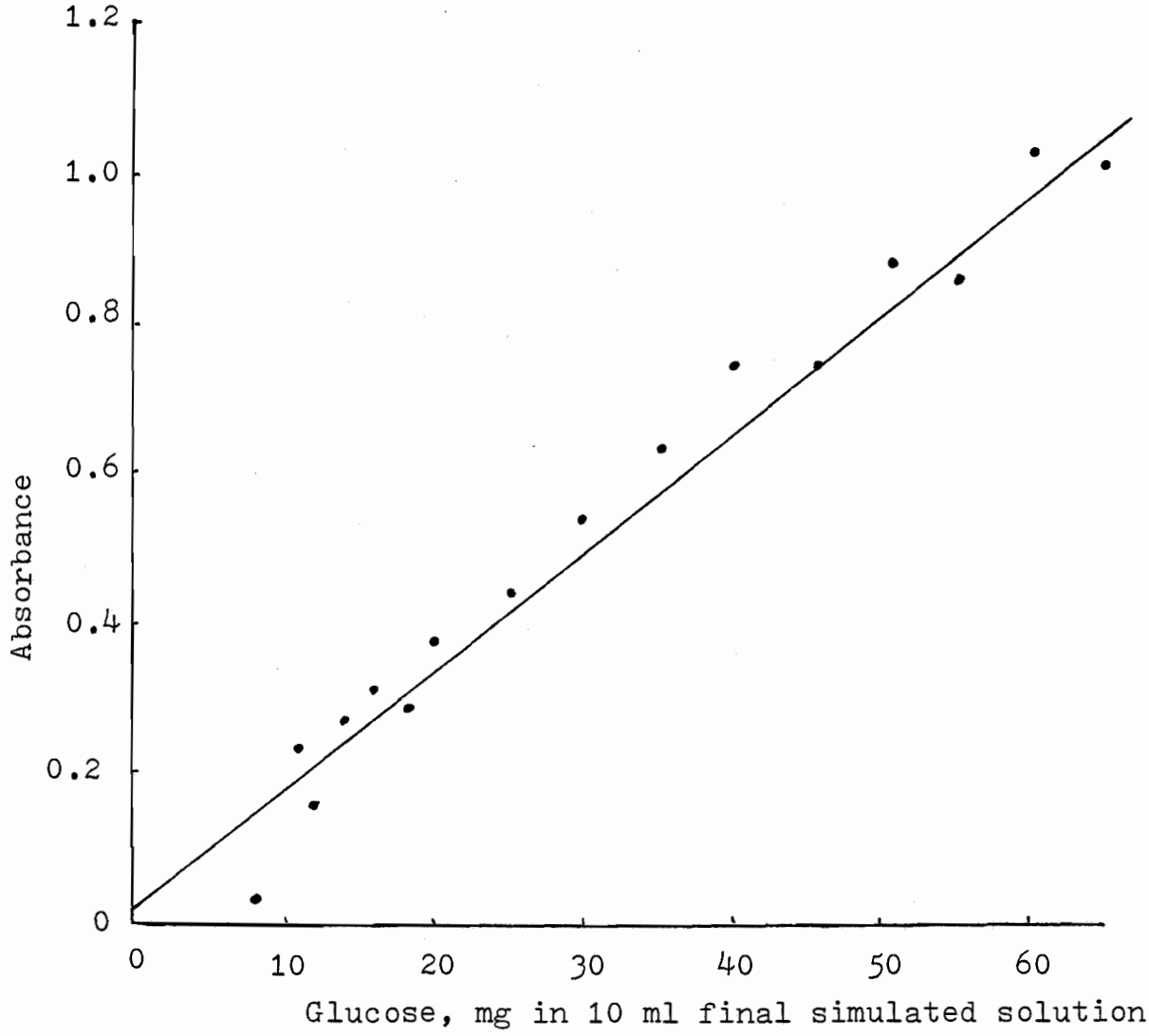


Figure 14. Examination of Beer's law of the reaction between D-glucose and the copper dye reagent.

X = weight ratio (glucose to internal standard)

independent variable

Figure 15 shows that the graph plotted was not linear. The intercept can be interpreted by solving for X by setting the equation equal to zero :

$$0 = - 0.049257 + 1.42226\ X - 0.233502\ X^2$$

then $X = 3.4832 \times 10^{-2}$ or 6.056

Now if the weight of the internal standard was 10 mg, then the weight ratio $X = 3.4832 \times 10^{-2}$ or 6.056 which means 0.3483 mg or 60.56 mg glucose is in 5 ml of the hydrolyzate out of 250 ml. Since, Y (area ratio) was zero which meant no glucose peak could be detected, then a glucose content under 0.3482 mg in 5 ml of hydrolyzate could not be detected. Therefore, the minimum amount of glucose in this solution that was detectable was 0.3482 mg (60.56 mg was not reasonable).

For mannose (Figure 16) the best fitting curve was found to be :

$$Y = 1.2244\ X^{1.2988}$$

or $\ln Y = 0.2044 + 1.2988 \ln X$ $\qquad R^2 = 0.9347$

If it were treated as a linear relationship, the regression would be :

$$Y = 0.978\ X \qquad \text{with} \quad R^2 = 0.8464$$

The first expression is the better fitting and will be used. The reason that the data for mannose were scattered was

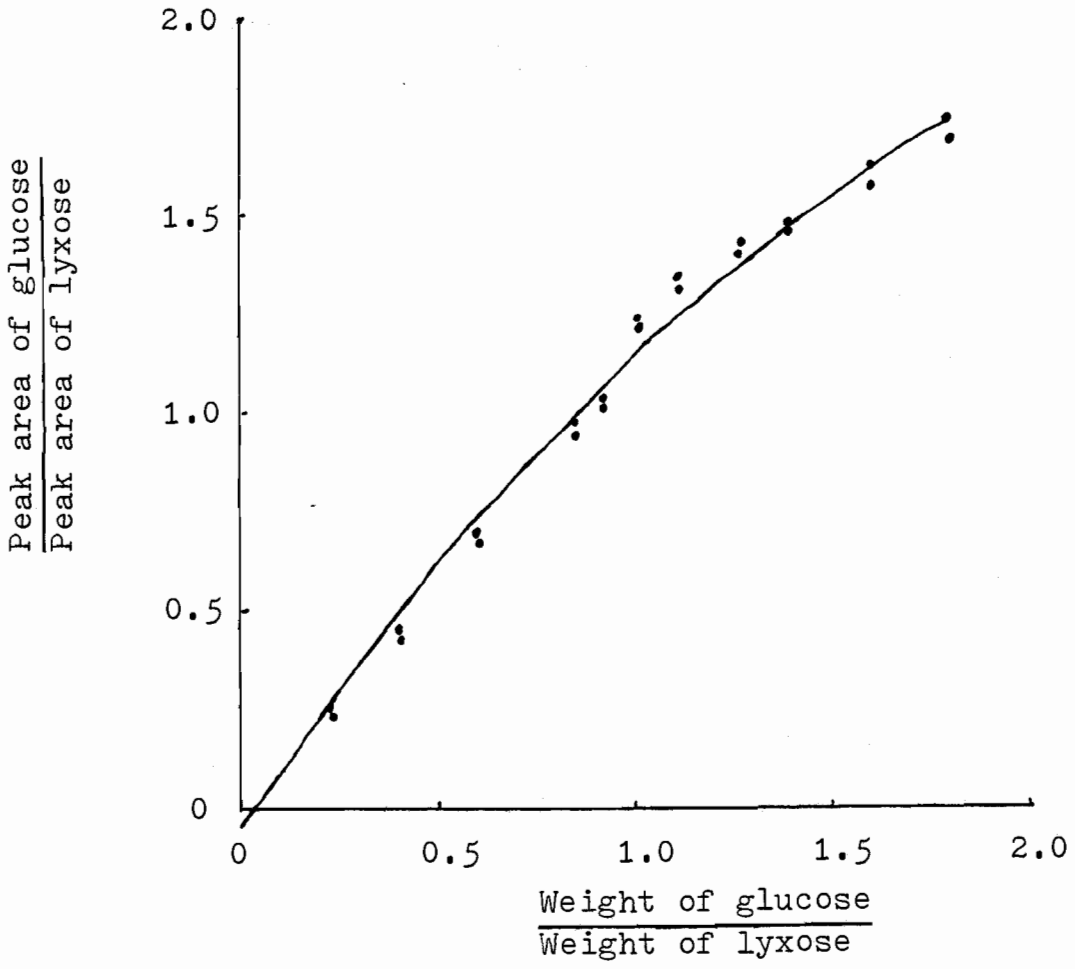


Figure 15. Recovery of authentic glucose to determine an instrument K factor with the borate buffer system.

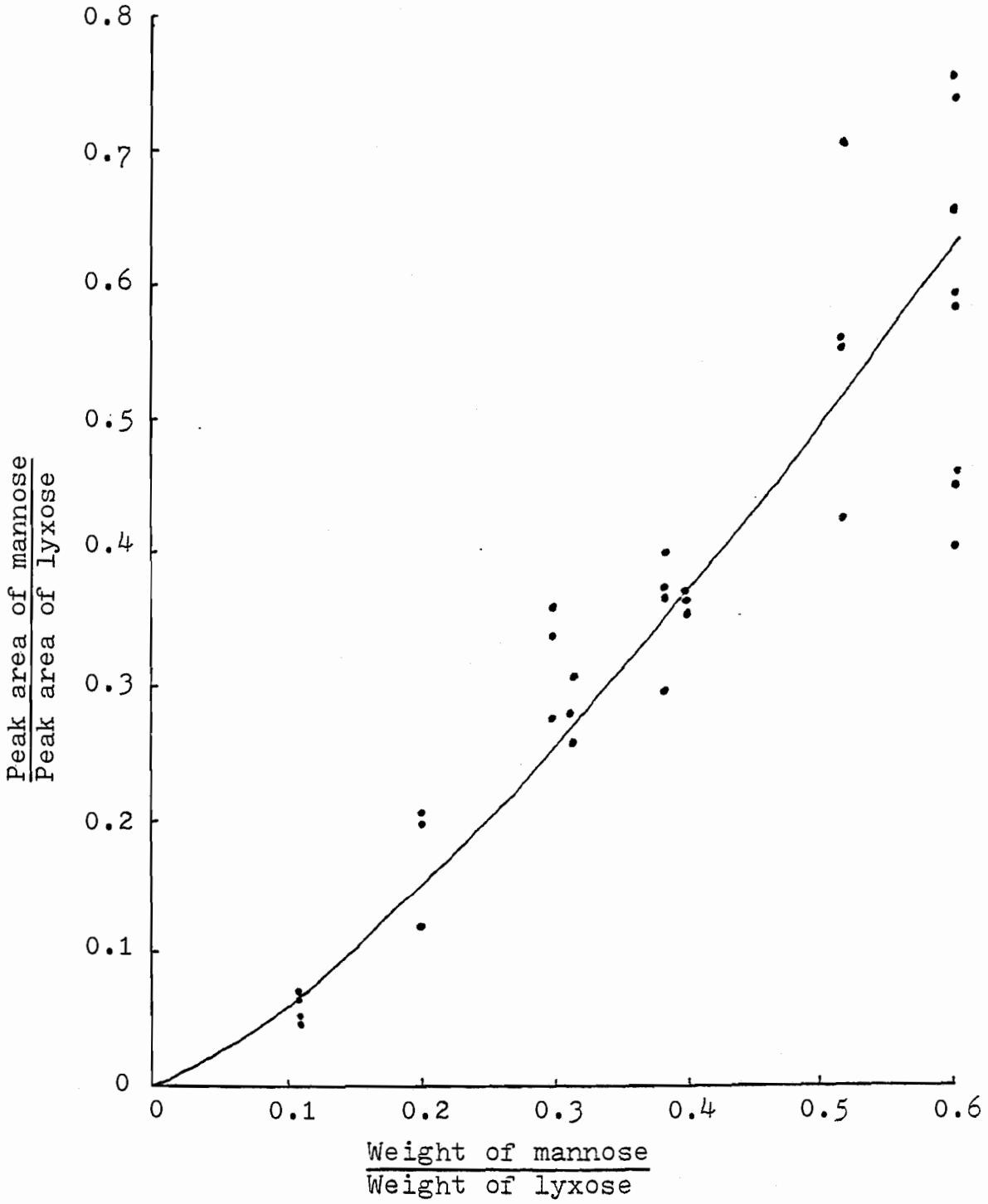


Figure 16. Recovery of authentic mannose to determine an instrument K factor with the borate buffer system.

that the range for mannose was small with a weight ratio up to only 0.6 compared with a weight ratio up to 2.0 for glucose. The ratio of weight for the mannose standard curve was small because these are the weights usually found in cellulosic hydrolyzates.

B. Hydrolysis of Polysaccharide Samples

1. Dissolution and Primary Hydrolysis

Cellulosic materials must be dissolved in order for hydrolysis to proceed. However, cellulose is quite insoluble in most solvents and in aqueous systems in particular. The original Monier-Williams technique (24) of dissolving the cellulosic materials in concentrated sulfuric acid and then hydrolyzing the polysaccharides with a dilute solution of the same acid has worked well. With this method the polymers never need to be transferred from one solvent to another. Water is simply added to dilute the acid before refluxing is begun.

The addition of the 77% sulfuric acid slowly to the cellulosic samples, which was in the form of a powder, was more convenient and easier to control that the opposite way of adding the cellulosic powder to the acid. Continuous stirring with a glass rod was also found to be a more effective means to dissolve the cellulose than to periodically evacuate the flask.

Purified cotton cellulose, when dissolved in 77% sulfuric acid, was never found to be completely colorless nor transparent. A light-brown color usually resulted. Dissolving the sample at ice-bath temperature to eliminate the possibility of burning the sample with acid did not reduce the brown color. The sample was very sticky and viscous when first dissolved. Then it became thinner and more dilute in appearance, which meant the dissolution was nearing completion. One hour of standing was found to be necessary to ensure complete dissolution. If dissolution was not complete, a white, flocculent precipitate formed upon dilution with water. When this occurred the sample was discarded and another one started.

2. Total Hydrolysis

The dissolved sample was diluted to a total volumn of 128 ml with distilled deionized water, which is calculated to be 2.84% in sulfuric acid. Cold distilled deionized water (50 ml) was first poured into a 250-ml round bottom flask. The sample solution was then poured slowly into it with shaking of the flask. Additional water (125 ml) was added afterward. A clear, nearly colorless solution was usually obtained with purified cellulose. The use of warm water or not shaking the flask well during dilution with water could result

in local heating with some degradation of the sample which would mean a low yield of glucose.

3. Kinetic Study of the Hydrolysis of Purified Cellulose

The relationship between recovery yield and refluxing time was reported by Laver, Root, Shafizadeh, and Lowe (21). Although Laver, Root, Shafizadeh, and Lowe (21) found that 4.5 hr of refluxing was sufficient for maximum sugar yield, the present work indicated a need for 5.5 hr of refluxing. The major difference between the two approaches is in the analyses of the sugars. Laver, Root, Shafizadeh, and Lowe (21) simply measured the total sugars released during hydrolyses by a copper reduction method. The method did not differentiate between glucose, galactose, mannose, arabinose and xylose. They also worked with pulp samples, not purified cellulose.

In the present work the analysis is of a purified cellulose which was shown to contain no other sugars. The borate buffer system was capable of separating and quantitatively measuring each monosaccharide. However, the spectrum showed the presence of glucose only (Figure 17). Figure 18 shows the release of glucose as refluxing is continued. It has the same general shape as that reported by Laver, Root, Shafizadeh, and Lowe for pulps (21). However, sugar degration

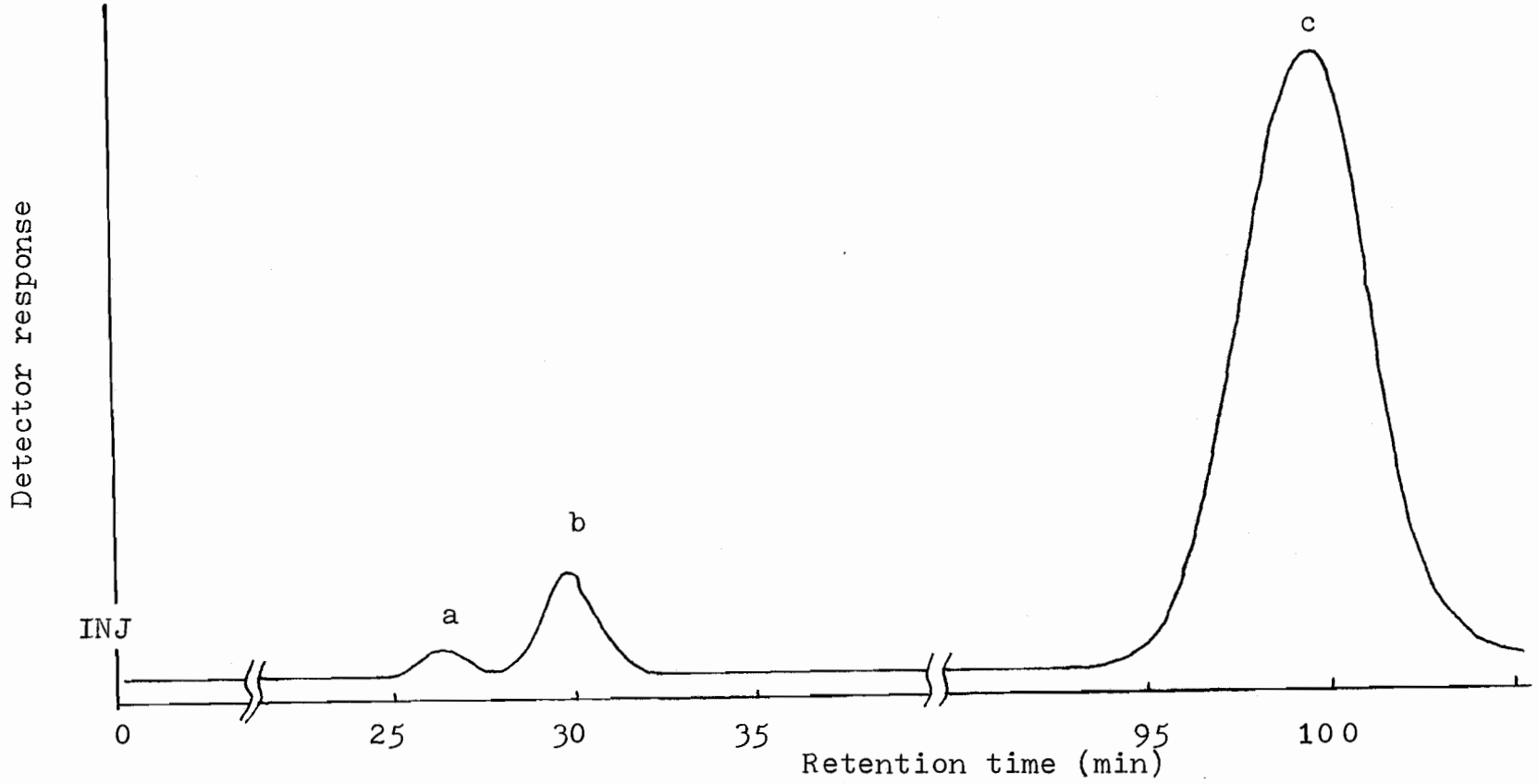


Figure 17. Liquid chromatographic spectrum of the acid hydrolyzate (refluxed 4 hr) of purified cellulose. Peaks "a" and "b" are from possible unhydrolyzed oligomers, peak "c" is from glucose. Conditions: column, Chromex DA-X4-11; eluant, 0.13 M potassium borate buffer; flow rate, 0.2 ml/min; detection, copper dye and absorbance monitor set at 546 nm.

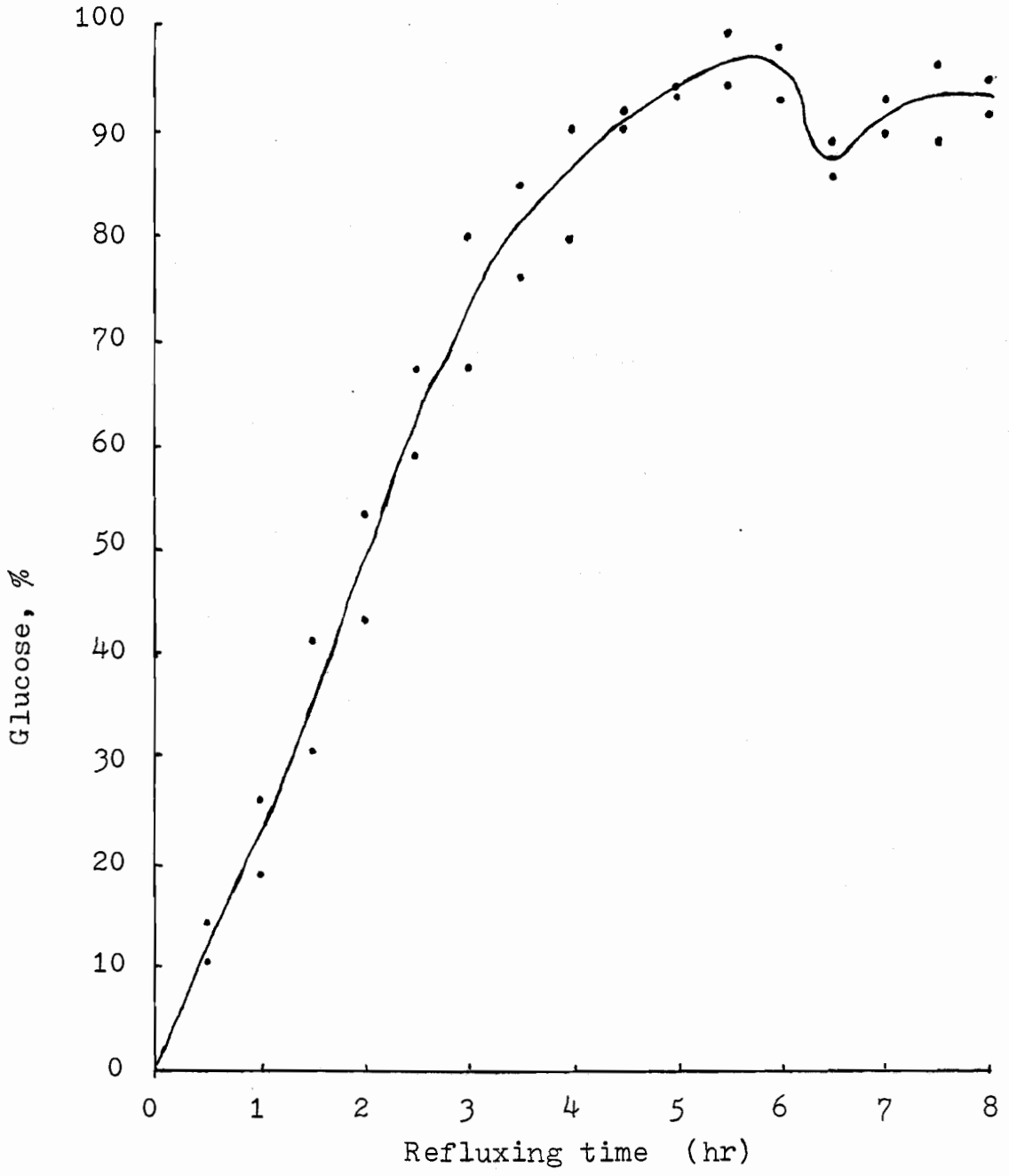


Figure 18. Hydrolysis of purified cellulose with refluxing sulfuric acid.

is less because there are no pentoses present which are acid sensitive. Glucose is reasonably resistant to acid degradation and so the maximum yield was excellent. The standard curve shown in Figure 15 was used to calculate the D-glucose yield.

C. Analyses of Cellulosic Samples by Using the Borate Buffer System

L-rhamnose was used as an internal standard for the analysis of six commercial pulp samples. L-rhamnose was used because it could not be found in the acid hydrolyzates of the samples. It was found later that there was still a slight chance of finding L-rhamnose in other hydrolyzates of wood materials. To avoid this uncertainty in future analysis, D-lyxose was chosen as the internal standard for the analysis of a commercial thermomechanical pulp sample. The instrument K factor of each monosaccharide was obtained : D-glucose 1.21, D-galactose 0.82, D-mannose 0.79, L-arabinose 0.70, D-xylose 1.09. The glucose content of the six pulp samples were 80.0%, 94.3%, 88.6%, 99.6%, 90.5%, and 108.0% on a weight yield basis. The D-mannose contents were 1.0%, 0.5%, 0.1%, 0.4%, 0.4% and trace. The D-xylose contents were 5.9%, 4.3%, 0.9%, 0.4%, 1.1% and 0.8%. The other monosaccharides were found in trace amounts only (Figure 19).

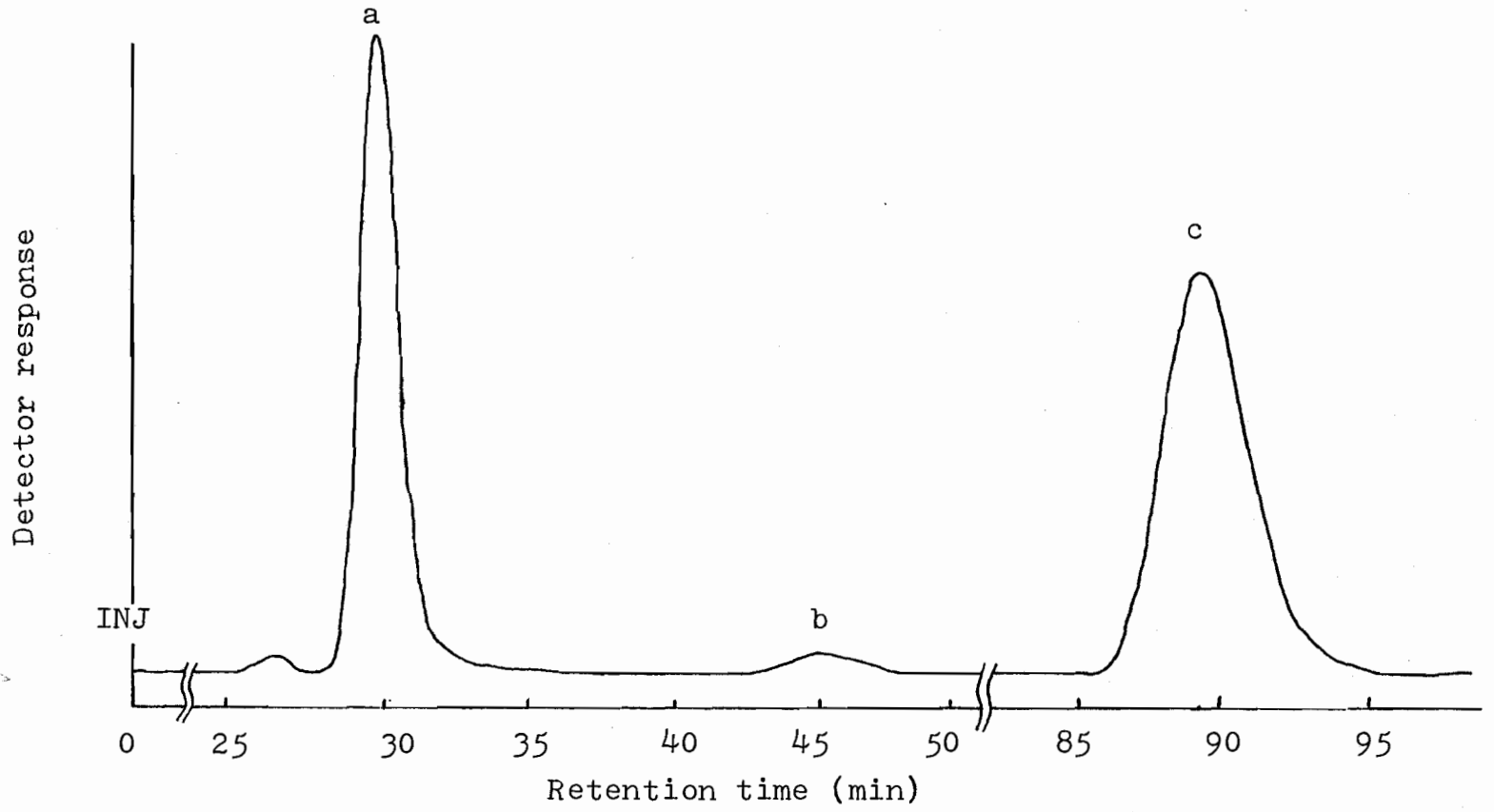


Figure 19. Liquid chromatographic spectrum of a typical acid hydrolyzate of a commercial pulp sample. Peak"a" is from rhamnose (internal standard), peak "b"is from mannose, peak "c" is from glucose. Conditions: column,Chromex DA-X4-11; eluant,0.13 M potassium borate buffer; flow rate, 0.2 ml/min; detection, copper dye and absorbance monitor set at 546 nm.

The acid insoluble lignin contents were low, from 0.33% to 0.35%.

Analysis of the thermomechanical pulp (Figure 20) showed 50.60% of the weight of the sample was glucose. The mannose content was 17.06% and the acid insoluble lignin was 29.41%.

It was found to be very important to keep the weight ratio of the estimated glucose in the sample to the internal standard in the range where the standard curve was established. Any extrapolation beyond the determined range would cause serious troubles. It was also noticed that the conditions of the instrument must be kept the same while running the samples as running the standard solutions. All of these reasons lead to the conclusion that a standardized procedure must be established from primary hydrolysis to liquid chromatographic analysis in order to get an accurate result.

D. Comparison of the Two Systems

The eluant solvent used in the A system was prepared by mixing two solvents, acetonitrile and water in a volume ratio 85:15. However, for the B system, weighing and dissolution problems were usually encountered while preparing the solution. Since the mixed copper dye reagent was not stable over long periods of time, a new batch of reagent had always to be made up. The time required to bring the system to equilibrium

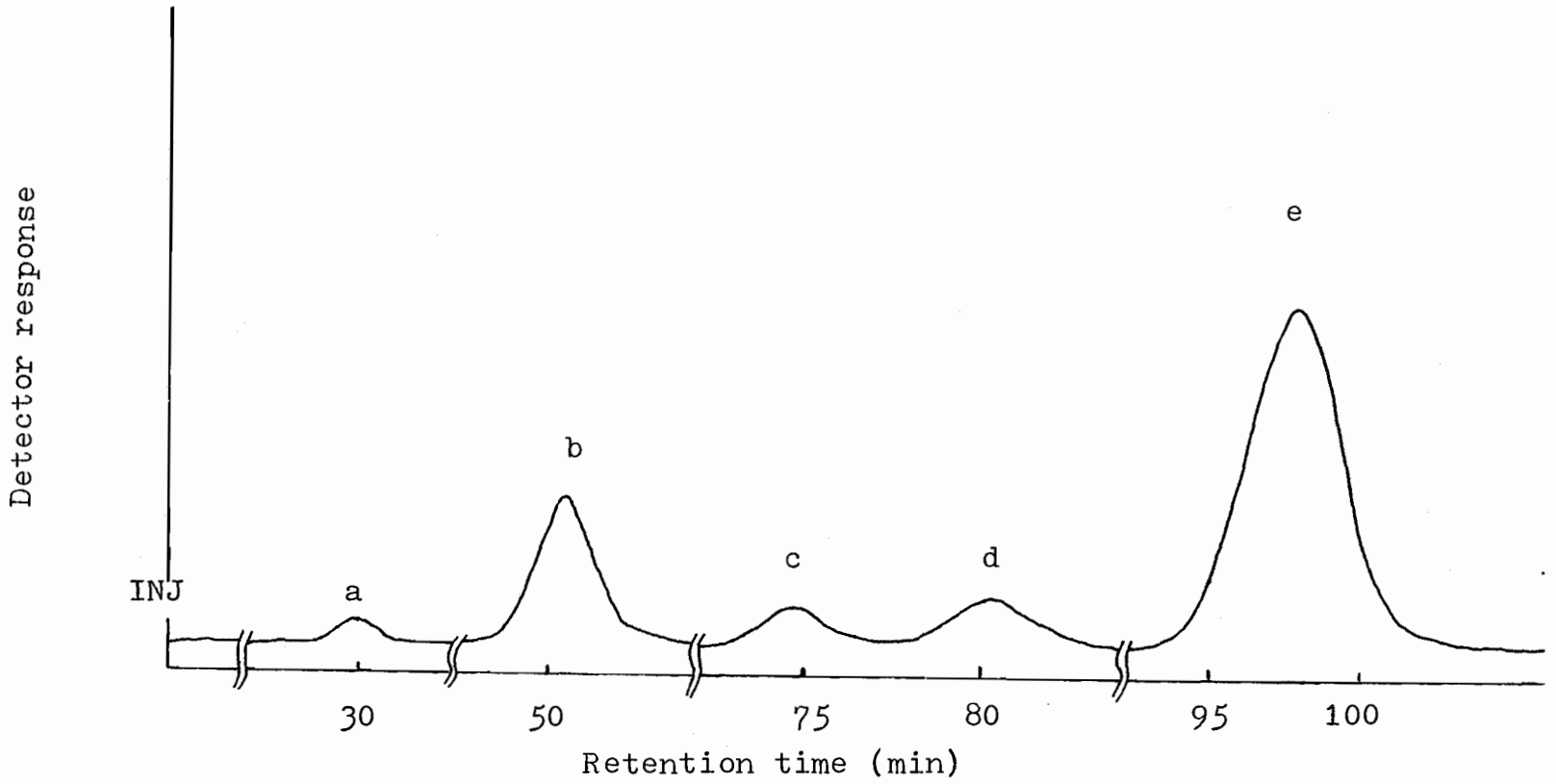


Figure 20. Liquid chromatographic spectrum of an acid hydrolyzate of a commercial thermomechanical pulp sample. Peak "a" is from a possible unhydrolyzed oligomer, peak "b" is from mannose, peak "c" is from galactose, peak "d" is from xylose, peak "e" is from glucose. Conditions: column, Chromex DA-X4-11; eluant, 0.13 M potassium borate buffer; flow rate, 0.2 ml/min; detection, copper dye and absorbance monitor set at 546 nm.

was long and the offset absorbance of the absorbance detector had to be adjusted before each use due to the spontaneous reaction of the solution. The column had to be kept at constant temperature and the boiling water bath sometimes was troublesome. The Teflon tubing could not withstand a pressure over 700 psi. If any crystallization occurred in a joint or in the detector, the pressure in the system would build up and the tubing would burst. When this occurred, a new set of tubing had to be used or a new connector had to be put in the line.

Excessive backpressure often developed in the column which ultimately resulted in complete blockage of the column. Usually it would damage the resin bed permanently and repacking of the column became necessary.

The monosaccharides, after separation by the column, were destroyed by reaction with the copper dye reagent and thus it was impossible to recover samples for further analysis such as NMR and IR if desired.

The advantages of system B over system A are : (a) The acid hydrolyzate does not have to be neutralized, it can be diluted and injected directly into the instrument. (b) The hydrolyzate does not have to be concentrated as a consequence of neutralization. (c) Most importantly the monosaccharides released on hydrolysis of woody materials can be well separated and thus each sugar can be quantitatively analyzed.

The borate buffer system described in this work also has a number of advantages over the previously used alditol acetate method which involved gas-liquid chromatography. The alditol acetate method is time consuming because it involves concentrating the hydrolyzate followed by reduction with sodium borohydride. The resulting alditols have to be carefully dried previous to acetylation. The alditol acetate gas-liquid chromatographic method was never considered to be a routine analytical method.

Alternatively the borate buffer method requires no sugar derivation and no sample preparation other than dilution to a known volume. Providing that great care is taken to standardize every step of the analysis, the borate buffer method can be used for the routine analysis of each monosaccharide in an hydrolyzate of woody materials.

V. SUMMARY AND CONCLUSIONS

1. Dissolution of cellulosic samples can be readily accomplished by using 77% sulfuric acid and it was found that stirring and "squelching" of the sample with a glass rod was more effective than applying vacuum.
2. A kinetic study of the hydrolysis of purified cellulose by using the liquid chromatographic borate buffer system showed that the maximum recovery yield could be as high as 99%. This was reached by refluxing for 5.5 hrs.
3. The acetonitrile-water system was easy in solution preparation, column preparation, setting up the equipment and possessed a short running time. However, it was not used as a routine method because the separation of the peaks were not good.
4. The mechanism of detection for the borate buffer system was that the cupric ion was reduced to the cuprous ion by the monosaccharides released by the hydrolysis of polysaccharides. These monosaccharides were then separated by the resin in the column. The cuprous ion so produced then complexed with 2,2'-bicinchoninic acid and yielded a chelating complex which gave a lavender color with a maximum absorbance at 560 nm. The intensity of the absorbance was measured at 546 nm in this work to determine the amount of monosaccharides present when compared with a carefully weighed internal standard.

5. The copper dye reagent itself increased in absorbance at 546 nm with increased heating time.
6. It was also found that the relationship between the absorbance of the copper dye reagent - monosaccharide reaction and heating time was not linear.
7. Beer's law was obeyed over the range of sugar concentrations encountered in the hydrolysis of the samples in this work.
8. Lyxose was chosen as a better internal standard than rhamnose for it has not been reported in wood hydrolyzates. Standard curves were used to eliminate the undesirable effect caused by different detector responses. The setting of the equipment must be kept the same in running standard solutions and unknown samples.
9. The liquid chromatographic borate buffer system is a sensitive but fragile method for monosaccharide analysis. It can still be improved and remodeled for a more convenient and routine analytical method.

BIBLIOGRAPHY

1. Alexander, R. J., and J. T. Garbutt. Use of sorbitol as internal standard in determination of D-glucose by gas-liquid chromatography. Analytical Chemistry 37(2): 303-305. 1965.
2. Belue, Greg P., and Gary D. McGinnis. High pressure liquid chromatography of carbohydrates. Journal of Chromatography 97:26-31. 1974.
3. Bio-Rad Laboratories. Out of the woods. The Liquid Chromatographer:Number 4, pps. 1-2, May 1980.
4. Blair, Mary Grace, In Carbohydrates: Chemistry, Biochemistry, Physiology. ed. W. Pigman. New York, NY. Academic Press. 1957. p.60.
5. Borchardt, L. C.,and C. V. Piper. A gas chromatographic method for carbohydrates as alditol-acetates. Tappi 53:257-260. 1970.
6. Brower, H. E., J. E. Jeffery, and W. W. Folsom. Gas chromatographic sugar analysis in hydrolyzates of wood constituents. Analytical Chemistry 38:362-364. 1966.
7. Corbett, W. M. Purification of cotton cellulose. In Methods in Carbohydrate Chemistry. Vol.III. ed. Roy L. Whistler. New York, NY. Academic Press. 1963. pps.3-4.

8. Crowell, E. P., and B. B. Burnett. Determination of the carbohydrate composition of wood pulps by gas chromatography of their alditol acetates. Analytical Chemistry 39:121-124. 1967.

9. Foster, A. B. Zone electrophoresis of carbohydrates. Advances in Carbohydrate Chemistry 12:81-115. 1957.

10. Friberg, T. S., E. E. Barnes, and R. E. Meyers. Carbohydrate compositions of wood pulps by high performance liquid chromatography. Weyerhaeuser Company, Tacoma, Washington. Personal communication. 1979.

11. Gershuns, A. L., A. A. Verezubove and Zh. A. Tolstykh. Photocolorimetric determination of copper with 2,2'-bicinchoninic acid. Chemical Abstract 55:14168g. 1961.

12. Gershuns, A. L., and V. L. Koval'. Spectrophotometric determination of copper with 2,2'-bicinchoninic acid. Analytical Abstract 21:53. 1971.

13. Giertz, H. W., Yellowing of paper. Svensk Papperstidn 48(13):317. 1945.

14. Gindler, E. M. Automated determination of glucose via reductive formation of lavender Cu(I)-2,2'-Bicinchoninate chelate. Clinical Chemistry 16:(6):519. 1970.

15. Gunner, S. W., J. K. N. Jones, and M. B. Perry. The gas-liquid partition chromatography of carbohydrate derivatives. Canadian Journal of Chemistry 39:1892-1899. 1961.

16. Hettinger, John and R. E. Majors. Separation of carbohydrates by high performance liquid chromatography. Varian Instrument Application (VIA) 10(2):6-7. 1976.

17. Jeffery, J. E., E. V. Partlow, and W. J. Polglase. Chromatographic estimation of sugars in wood cellulose hydrolyzates. Journal of Analytical Chemistry 32:1774-1777. 1960.

18. Johnson, Edward L. Liquid Chromatography Bibliography. Varian Associates. Inc. 1973. pps.253-259.

19. Ladisch, Michael R., Aronson L. Huebner, and George T. Tsao. High-speed liquid chromatography of cellodextrins and other saccharide mixtures using water as the eluant. Journal of Chromatography 147:185-193. 1978.

20. Ladisch, Michael R., and George T. Tsao. Theory and practice of rapid liquid chromatography at moderate pressures using water as eluant. Journal of Chromatography 166:85-100. 1978.

21. Laver, Murray L., D. F. Root, F. Shafizadeh, and J. C. Lowe. An improved method for the analysis of the carbohydrates of wood pulps through refined conditions of hydrolysis, neutralization and monosaccharide separation. Tappi 50:(12):618-622. 1967

22. McGinnis, G. D., and P. Fang. The separation of substituted carbohydrates by high-performance liquid chromatography. Journal of Chromatography 130:181-187. 1977.

23. McGinnis, G. D., and P. Fang. Separation of substituted carbohydrates by high-performance liquid chromatography. II. Journal of Chromatography 153: 107-114. 1978.

24. Monier-Williams, Gordon Wickham. The hydrolysis of cotton cellulose. Journal of the Chemical Society 119: 803-805. 1921.

25. Mopper, K., and E. M. Gindler. A new noncorrosive dye reagent for automatic sugar chromatography. Analytical Biochemistry 56:440-442. 1973.

26. Mustatin, J. S., N. S. Frumina, and V. S. Kovaleva. Determination of copper in various samples with 2,2'-bicinchoninic acid. Chemical Abstract 59:9307h. 1963.

27. Nakano Saburo. 2,2'-Biquinoline derivatives. VI. Copper (I) chelates of 4,4'-substituted 2,2'-biquinoline derivatives and the determination of copper by 2,2'-bicinchoninic acid. Chemical Abstract 58:3390e. 1963.

28. Palmer, James K. A versatile system for sugar analysis via liquid chromatography. Analytical Letters 8(3): 215-224. 1975.

29. Saeman, Jerome F., Janet L. Bubl, and Elwin E. Harris. The quantitative saccharification of wood and cellulose. United States Department of Agriculture Forest Service, U.S. Forest Products Laboratory Report No. R1458. 1944.

23. McGinnis, G. D., and P. Fang. Separation of substituted carbohydrates by high-performance liquid chromatography. II. Journal of Chromatography 153: 107-114. 1978.

24. Monier-Williams, Gordon Wickham. The hydrolysis of cotton cellulose. Journal of the Chemical Society 119: 803-805. 1921.

25. Mopper, K., and E. M. Gindler. A new noncorrosive dye reagent for automatic sugar chromatography. Analytical Biochemistry 56:440-442. 1973.

26. Mustatin, J. S., N. S. Frumina, and V. S. Kovaleva. Determination of copper in various samples with 2,2'-bicinchoninic acid. Chemical Abstract 59:9307h. 1963.

27. Nakano Saburo. 2,2'-Biquinoline derivatives. VI. Copper (I) chelates of 4,4'-substituted 2,2'-biquinoline derivatives and the determination of copper by 2,2'-bicinchoninic acid. Chemical Abstract 58:3390e. 1963.

28. Palmer, James K. A versatile system for sugar analysis via liquid chromatography. Analytical Letters 8(3): 215-224. 1975.

29. Saeman, Jerome F., Janet L. Bubl, and Elwin E. Harris. The quantitative saccharification of wood and cellulose. United States Department of Agriculture Forest Service, U.S. Forest Products Laboratory Report No. R1458. 1944.

30. Saeman, Jerome F., Wayne E. Moore, Raymond L. Mitchell, and Merrill A. Millett. Techniques for the determination of pulp consitituents by quantitative paper chromatography. Tappi 37:(8):336-343. 1954.

31. Saeman, Jerome F., Wayne E. Moore, and Merrill A Millett. Hydrolysis and quantitative paper chromatography. In Methods in Carbohydrate Chemistry Vol.III. ed. Roy L. Whistler. New York, NY. Academic Press, 1963. pps 54-69.

32. Sawardeker, J. S., and J. H. Sloneker. Quantitative determination of monosaccharides by gas-liquid chromatography. Analytical Chemistry 37:(7):945-947. 1965.

33. Sawardeker, J. S., J. H. Sloneker, and A. Jeanes. Quantitative determination of monosaccharides as their alditol acetates by gas-liquid chromatography. Analytical Chemistry 37:1602-1604. 1965.

34. Sinner, M., M. H. Simatupang, and H. H. Dietrichs. Automated quantitative analysis of wood carbohydrates by borate complex ion-exchange chromatography. Wood Science and Technology 9:307-322. 1975.

35. Sinner, M., and J. Puls. Non-corrosive dye reagent for detection of reducing sugars in borate complex ion-exchange chromatography. Journal of Chromatography 146:197-204. 1978.

36. Waters Associates. μ BONDAPAK and μ PORASIL liquid chromatography column care and use manual. page 3-1. March 1976.

37. Weigel, H. Paper electrophoresis of carbohydrates. Advances in Carbohydrate Chemistry 18:61-97. 1963.

38. Whatman. Separations on Whatman media. HPLC Reference List No.1 Interim Technical Data. Whatman Inc. Chemical Separation Division. Technical Bulletin ITD-131. page 5. October 1979..

39. Wolfrom, M. L., D. L. Patin, and Rosa M. DeLeaderkremer. Thin-layer chromatography on microcrystalline cellulose. Journal of Chromatography 17:488-494. 1965.