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AN ABSTRACT OF THE THESIS OF Minoo Shakerin Sweet for the Master of Science in Chemistry presented May 17, 1979.

Title: The Concentration and Speciation of Sugars in Natural Waters

APPROVED BY MEMBERS OF THE THESIS COMMITTEE:

Edward M. Perdue, Chairman

Dennis W. Barnum

Alfred S. Levinson

Due to the importance of carbohydrates in biological systems, many efforts have been made to develop a quantitative method for analysis of carbohydrates in natural waters. The low concentrations of dissolved sugars in natural waters require a sensitive analytical method. In this study, gas chromatography of alditol acetate derivatives of sugars was investigated for quantitative and qualitative analysis of individual dissolved sugars in natural waters. The alditol acetate derivatives of sugars give only one derivative for each sugar, yielding qualitative and quantitative results.

The detection limit was 25 nM for each sugar. Because of this very low detection limit, only 100 ml of sample was required for analysis.

From measurements of the alditol acetate derivatives, qualitative and quantitative analysis of pentoses (arabinose and xylose) and hexoses (mannose, galactose, and glucose) were obtained from The Williamson River and its tributaries, which are located near Klamath Falls, Oregon. Total organic carbon concentrations vary greatly in this river system as a result of the river passing through Klamath Marsh, which introduces very high amounts of humic substances into the river system.

The range of total concentrations of dissolved sugars is 0.07 to 7.3 μM ; the lowest occurring in the spring waters, and the highest in humic-rich waters.

Monosaccharide, polysaccharide, and humic-bonded saccharide concentrations, which were obtained from three sample sites, showed very low concentrations of monosaccharides, moderate concentrations of polysaccharides, and moderately high concentrations of humic-bonded saccharides.

THE CONCENTRATION AND SPECIATION OF SUGARS
IN NATURAL WATERS

by
MINOO SHAKERIN SWEET

A thesis submitted in partial fulfillment of the
requirements for the degree of

MASTER OF SCIENCE
in
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1979

TO THE OFFICE OF GRADUATE STUDIES AND RESEARCH:

The members of the Committee approve the thesis of
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INTRODUCTION

An important study of the composition of organic matter in natural waters was conducted by Birge and Juday (1926, 1934), who studied over 500 Wisconsin lakes. The composition of the dissolved organic matter was estimated to be 15.6 percent crude protein, 0.7 percent lipid material, and 83.7 percent carbohydrate. Neither the protein nor the carbohydrate concentrations were measured analytically, but rather it was assumed that all nitrogen was present as crude protein (percent protein is approximately 6.25 times the percent nitrogen). The remaining water-soluble organic material was assumed to be carbohydrates.

In 1957, Shapiro found that the ethyl acetate extractable organic matter from natural waters was a mixture of colorless and colored carboxylic acids. The chemical tests showed that these organic acids have a phenolic or enolic character. Infra-red spectroscopy of various derivatives indicated a mixture of hydroxy carboxylic acids. The dissolved organic matter from eleven lakes had reasonably constant composition. According to Shapiro (1957), the dissolved organic matter (which was very resistant to chemical oxidation) probably originated as a result of decomposition of organic matter in soil and possibly in sediments as well.

From 1957 until recent years, the study of dissolved organic matter in natural waters has been continued. The distribution of some organic material has been investigated in several lake systems (Saunders, 1963; Walsh, 1965; and Weinmann, 1970). Leenheer and Malcolm (1973) fractionated dissolved organic matter by free-flow

electrophoresis and found significant amounts of colorless organic material in water and soil samples. Leenheer and Huffman (1976) developed an adsorption procedure which utilizes macrorecticular resins to separate dissolved organic matter into a hydrophilic and a hydrophobic fraction. The hydrophilic organic solute was fractionated into acid, base, and neutral compounds. Reuter and Perdue (1977) reported that the bulk of dissolved organic matter in natural waters consists of highly oxidized, chemically and biologically stable polymeric compounds closely resembling soil humic substances. The aquatic humic polymers participate in complex formation through ionizable functional groups.

Carbohydrates from a variety of biological and environmental sources have been measured and analyzed. There are numerous studies on carbohydrates in soil (Lowe, 1968, 1969, 1975; Clark and Tan, 1969), and in river and lake sediments (Handa and Mizuno, 1973; Handa, 1972; Vallentyne and Bidwell, 1956; Whittaker and Vallentyne, 1957). Carbohydrates have also been measured in aquatic plants (Rogers, 1965).

In natural waters, carbohydrates have not been studied as extensively as in sediments. Vallentyne and Whittaker (1956) analyzed free sugars in filtered lake water by paper chromatography. They could not state any exact amount of dissolved free sugars, but they did state that the concentration of free dissolved sugars was a few $\mu\text{g/l}$. In most studies since 1956, total hydrolyzable carbohydrates have been measured using the colorimetric method of phenol-sulfuric acid (Dubois et al., 1956) in which the total carbohydrate concentration is reported in glucose equivalents. This colorimetric method is based

on the formation of an orange-yellow color which is produced by reaction of the sugar with phenol-sulfuric acid reagent. The wavelength of maximum adsorption for hexoses is at 490 nm and for pentoses at 480 nm. Therefore, if the total sugar analysis is done, there would be a problem caused by having two maximum wave lengths which are close together, making quantitative analysis difficult. The gram adsorptivity is different for equal concentrations of different sugars, with absorbance ranging from 0.67 to 0.97 for .44 μ moles of each sugar. The calibration curve was obtained using 5 to 0.19 mM of each sugar. The method described by Dubois et al. (1956) can be used to give an estimation of the sugar content of pure solutions. In more recent years, the method of Dubois et al. (1956) has been used for determination of total dissolved carbohydrates in natural waters in the presence of humic substances (Bikbulatov and Skopintsev, 1974). The amounts of sugars in some natural waters before and after hydrolysis, with 2 N H_2SO_4 for six hours was obtained. After hydrolysis, the concentration of total sugar varied from 10 to 20 μ M, which was similar to the values obtained without prior hydrolysis (9 - 23 μ M). The concentrated sulfuric acid used in this method can apparently hydrolyze the carbohydrates in natural waters at the same time the colored derivative is being formed. The wavelength used for the colorimetric method of Bikbulatov and Skopintsev (1974) was 485 nm, which is between the absorbance maxima of hexoses (490 nm) and pentoses (480 nm) obtained by Dubois et al. (1956).

Several paper chromatographic methods have been used for determination of individual carbohydrate concentrations in natural waters

(Vallentyne and Whittaker, 1956; and Degens et al., 1964). These methods are cumbersome and time consuming due to the large volume of water sample (2,000 ml) which must be evaporated to dryness. Large volumes of water are required because the concentrations of dissolved carbohydrates are very low in natural waters. The detection limit for the paper chromatographic method is 1 μg for each sugar. Using this method, Degens and Reuter (1964) obtained 0.08 - 0.19 μM for the concentrations of free sugars in offshore California seawaters.

Semenov et al. (1967) determined free reducing sugars and sugars formed upon hydrolysis of more complex carbohydrate-like compounds in the waters of some Soviet rivers by using the p-aminohippuric acid method (Ivleva et al., 1964). The concentration of free sugars obtained by Semenov et al., (1967) was 0.17 - 1.11 μM .

Enzymatic methods have also been applied to the analysis of sugars in natural waters, but are useful only for glucose and, to some extent, for galactose (Swain et al., 1967).

Gas chromatography of trimethylsilyl ether derivatives of sugars has been used for analysis of individual sugars in natural waters (Henning, 1977). The main sugars found in hydrolyzed lake samples were glucose, galactose, xylose, rhamnose, fructose, and fucose. These sugars were present as macromolecules, not as free sugars. The annual carbohydrate carbon content was 0.70 μM .

The main purpose of the project described herein was to investigate the use of alditol acetate derivatives for qualitative and quantitative analysis of dissolved carbohydrates in natural waters. During the writing of this thesis, an unpublished paper in which a similar

method was used was presented by N. Handa at the 177th A.C.S. National Meeting in Honolulu, Hawaii.

The research described herein is part of a more comprehensive study of the impact of marsh environments on the distribution and transport of organic matter in natural waters. The Williamson River, which is located in southern Oregon, was chosen for this study because of the large change in total organic carbon (TOC) which occurs when this river flows through Klamath Marsh (Figure 1). The high concentration of humic substances in this river, after passing through the marsh, made this river system very interesting for the investigation of the relationship between humic substances and carbohydrates in the water.

The Williamson River and its tributaries exhibit a great diversity of biological and geological environments. The river originates from underground springs, then flows for about twenty miles through an area which is used extensively for ranching. Consequently, a significant amount of nutrients enter the stream, increasing algal productivity. Then the river flows through Klamath Marsh, which has a high concentration of humic substances. Downstream from the marsh, the river contains very high concentrations of humic substances, with some decrease in TOC with increasing distance from the marsh. Then Spring Creek, which is almost free of humic substances, flows into the Williamson River, thus diluting the TOC concentration in the river. Sprague River has an entirely different drainage basin from the Williamson River; however, both are located in the same general area and have similar temperature and rainfall. When the Sprague River merges with the Williamson River, an even greater dilution

of TOC occurs. Finally, the Williamson River flows into Upper Klamath Lake, which is regarded as the most highly eutrophic lake in Oregon.

Figure 1. Location of sample sites in the Williamson River Basin.

EXPERIMENTAL

ANALYSIS OF STANDARD SUGAR SOLUTIONS

The first objective was to prepare alditol acetate derivatives of individual standard sugars (see Appendix) in the mixture of standard sugars.

Preparation of Standard Sugar Stock Solutions

A concentrated stock solution of mannose, galactose, glucose, arabinose, and xylose, each at a concentration of 5,000 μM , was prepared. The dilute stock sugar solution was made by a 20-fold dilution of the 5,000 μM solution in order to obtain a concentration of 250 μM for each sugar. The stock solutions were prepared on the same day that samples were collected from the river. The dilute stock solution, which contained 0.03 M NaN_3 as a preservative, was kept in the refrigerator at 5°C.

Derivatization Procedure

For making standard sugar derivatives, 100, 200, and 400 μl of 250 μM sugar stock solution were pipetted in graduated Reacti-Vials (a 3 ml, thick glass bottle with the internal cone shape and Teflon-lined cap, which was obtained from Pierce Chemical Company). The volume in each Reacti-Vial was adjusted to 0.4 ml, and the pH of each solution was brought to approximately pH 8 by addition of aqueous NaOH. For reduction of sugars to their alditols (Abdel-Akher and Hamilton,

1951), 0.4 ml of 0.5 N NaBH_4 solution were added to each vial. The solutions were allowed to stand at room temperature for one hour, which was sufficient time for the reduction of the sugars if the amount of NaBH_4 used is much greater than the sugars. After one hour, the excess of NaBH_4 was decomposed with dropwise addition of glacial acetic acid until evolution of hydrogen gas ceased, followed by adding one drop of 6 N HCl to prevent complex formation between alditols and borate ions (Boeseken, 1949), which will interfere with the derivatization process. The solutions in the vials were evaporated to dryness on a hotplate at 80°C under a stream of filtered air. The residues were allowed to cool to room temperature and then 1 ml of MeOH-HCl (0.1 ml of 12M HCl in 100 ml of absolute MeOH) was added to each residue for dehydration. In this step, borate ions in the presence of methanol will form trimethylborate, which volatilizes from solution (Crowell, 1967). The solutions were then evaporated to dryness by the method described previously. The MeOH-HCl treatment was repeated twice. One ml of absolute ethanol was added to the residue and it was centrifuged at 2400 rpm for two minutes. The supernatant solution was decanted into a clean vial and evaporated to dryness. The dried residue thus obtained was placed over P_2O_5 in a dessicator for at least three hours.

Acetylation of alditols was achieved by adding 0.2 ml of redistilled pyridine and 0.2 ml of acetic anhydride to the dried alditol residues, and then heating in a sealed Reacti-Vial with a Teflon-lined cap over a hotplate at 100°C for 15 minutes. The solution was allowed to cool to room temperature and 0.2 ml of distilled

water was added to destroy the excess acetic anhydride. The solution was then evaporated to dryness at 70°C. Alditol acetates were dissolved in chloroform solution containing inositol acetate as an internal standard, by adding 200 μ l of 250 μ M inositol acetate in chloroform. All the vials were capped with Teflon-lined caps and the samples were then ready to be injected into the gas chromatograph. At this point, each Reacti-Vial contained 25, 50, or 100 nmoles of each standard sugar derivative and 50 nmole of inositol acetate.

Gas Chromatographic Method

A Hewlett-Packard 5750B gas chromatograph with a flame ionization detector was used with nitrogen as the carrier gas. The detector signal was connected to a Hewlett-Packard chart recorder (model 7127A) and a Hewlett-Packard 3380A integrator.

All conditions were as follows:

column	6 ft. x 2 mm I.D. glass column (O.D. $\frac{1}{4}$ in.), packed with 3% SP-2330 on 100/120 Supelcoport (Cyano silicone on 100/120 mesh acid-washed dimethylchlorosilane treated diatomite, obtained from Supelco Company).
column temperature	188°C isotherm for 4 min. 188°C - 210°C at 2°C/min. 210°C isotherm for 5 min.
injector temperature	220°C
detector temperature	270°C
carrier gas flow rate	30 ml/min.
hydrogen flow rate	32 ml/min.
air flow rate	47 ml/min.
attenuation	10 x 4

syringe	Hamilton, 5 μ l
injection volume	1 - 2 μ l
recorder chart speed	0.25 in./min.

Calibration Curves

The standard derivatives contained 25, 50, and 100 nmoles of each sugar, and all of them contained 50 nmoles of inositol acetate as an internal standard. Under conditions described earlier, the gas chromatogram of the standard derivatives was obtained (Fig. 2). The area of each peak was calculated by the integrator, and the ratio of each peak area to the peak area of the internal standard was calculated (Table 1).

The ratios of areas vs. amounts for each sugar were plotted (Figs. 3 and 4). They showed very good linearity (correlation coefficients ranged from 0.9979 to 0.9998).

The detection limit was 2.5 nmoles and the derivatization procedure for many sets of standards showed very good reproducibility.

- 1 Arabinitol acetate
- 2 Xylitol acetate
- 3 Mannitol acetate
- 4 Galactitol acetate
- 5 Glucitol acetate
- 6 Inositol acetate

The standard used in this chromatogram contained 100 nmoles of each alditol acetate and 50 nmoles of inositol acetate in 0.2 ml of CHCl_3 .

Figure 2. Gas chromatogram of standard derivatives.

TABLE I

CALIBRATION OF THE ALDITOL ACETATE METHOD

Peak Area of Sugar / Peak Area of Internal Standard

<u>nmoles of sugar</u>	<u>Arabinose</u>	<u>Xylose</u>	<u>Mannose</u>	<u>Galactose</u>	<u>Glucose</u>
25	0.41	0.42	0.48	0.42	0.47
	0.44	0.44	0.53	0.46	0.48
50	0.78	0.75	0.86	0.78	0.92
	0.80	0.76	0.87	0.78	0.92
100	1.43	1.43	1.66	1.51	1.83
	1.46	1.44	1.69	1.53	1.80

Each standard was measured twice.

Each standard contained 50 nmoles of inositol acetate as the internal standard.

Figure 3. Calibration curve for standard pentoses

Figure 4. Calibration curves for standard hexoses

ANALYSIS OF RIVER SAMPLES

The analysis of total dissolved sugars in river water samples involved several preparative steps prior to derivatization (Fig. 5). After hydrolysis of dissolved polymeric saccharides in 3 N HCl, humic substances (which might interfere with the derivatization) were removed from solution by adsorption on XAD-7 resin (a macrorecticular acrylic ester, which was obtained from Rohm and Haas).

Unfractionated Samples (UF)

One hundred ml of each sample were filtered through a 0.45 μ m Millipore filter (pre-washed with 1,000 ml of distilled water). Each 100 ml filtered sample was poured into a glass bottle (150 ml), and concentrated on a hotplate at 80°C under a stream of filtered air to a final volume of ~1 ml. All concentrated water samples were transferred from the glass bottles to 5 ml Reacti-Vials. The glass bottles were rinsed with distilled water and the rinsings were transferred quantitatively into the vials. The samples were subsequently concentrated to ~0.2 ml.

The volume of each sample was adjusted to 0.3 ml in a graduated Reacti-Vial. Then 0.3 ml of 6 N HCl were added to each vial in order to obtain a 3 N HCl concentration for hydrolysis. Hydrolysis was accomplished by heating the above samples in sealed Reacti-Vials at 110°C for one hour. After hydrolysis, the samples were allowed to cool to room temperature.

Figure 5. Flow chart for analysis of unfractionated samples (UF).

The cooled hydrolysates were transferred to a 10 ml syringe which was connected to a Swinnex adaptor (a 13 mm diameter filter holder for filtering small volumes, which was obtained from Millipore) containing a glass-fiber filter (for removing any solid particles). The Reacti-Vials were rinsed twice into the syringe with 2.5 ml of 0.01 N HCl solution. All samples and washings were filtered directly onto a 4 ml XAD-7 column. They were then passed through the column and the column was washed with two 5 ml aliquots of 0.01 N HCl (it is necessary to use 0.01 N HCl for washing, because at pH higher than 2, the humic substances from the samples do not adsorb to the XAD-7 resin). The eluent was collected in a 50 ml beaker. At this point the volume of the sample was about 20 ml. These samples were concentrated to 1 ml on a hotplate at 90°C under a stream of filtered air. Each one ml of concentrated hydrolysate was transferred quantitatively from the beakers to the Reacti-Vials, and again were concentrated in the same way to a volume of 0.3 ml. The pH was adjusted to 8 by dropwise addition of 5 N NaOH (an acidic solution will interfere with the derivatization procedure). The final volume was adjusted to 0.4 ml. At this point, hydrolysates were ready for derivatization by the previously described method.

Fractionated Samples

In order to determine whether dissolved sugars were present as monosaccharides (MS), polysaccharides (PS), or humic-bonded saccharides (HS), analysis of fractionated samples was performed for unhydrolyzed and hydrolyzed fractions. Two hundred twenty ml of each river water sample were filtered through a 0.45 μ m Millipore filter (pre-washed

with 1,000 ml of distilled water). The filtrate was acidified to pH 1.6 - 2.0 by adding 0.3 ml of 6 N HCl. The acidified filtrate was passed through 4 ml of an XAD-7 column in order to remove humic substances (and humic-bonded saccharides). The first 20 ml of eluent were discarded. The eluent obtained was divided into two portions (Fig. 6) for analysis of unhydrolyzed and hydrolyzed fractions. Derivatization of the unhydrolyzed fraction yields only dissolved monosaccharides. Derivatization of the hydrolyzed fraction yields dissolved monosaccharides, polysaccharides, and some humic-bonded saccharides (the efficiency of adsorption of HS on XAD-7 is less than 100%).

Unhydrolyzed Fraction (F₁). A 100 ml aliquot of the eluent from the XAD-7 resin was neutralized with 0.3 ml of 6 N NaOH (to pH 7-8). The neutralized sample was evaporated to ~2 ml in a 150 ml glass bottle in the same way as was done for the unfractionated samples. The final volume after transferring from the glass bottle to the Reacti-Vial, rinsing the bottle, and evaporating again was then adjusted to 0.4 ml. The samples were now ready for derivatization.

Hydrolyzed Fraction (F₂). A 100 ml sample of the eluent from the XAD-7 column was concentrated to a volume of 0.3 ml, and then hydrolyzed by adding 0.3 ml of 6 N HCl and heating at 110°C for one hour in a sealed Reacti-Vial. The sample, after cooling to room temperature, was ready for derivatization.

Figure 6. Flow chart for analysis of fractionated samples.

FRACTIONAL RECOVERIES DURING ANALYSIS OF SUGAR SPECIES

Losses of saccharides occurred during different treatments (Figs. 5, 6) for different sugar species. Evaporation, hydrolysis and passing the sample through XAD-7 resin are the steps which are responsible for losses of sugars.

Recovery During Evaporation of Water Samples

The evaporation step of the experiment has shown losses of 5% for the monosaccharides. Air oxidation possibly converted the aldehyde group of a sugar to a carboxyl group. Since polysaccharides and humic-bonded saccharides do not contain an aldehyde group, their recoveries are assumed to be 100%. If the recovery during evaporation is called α^e , then recoveries for monosaccharides (α_m^e), polysaccharides (α_p^e), and humic-bonded saccharides (α_h^e) are:

$$\alpha_m^e = 0.95 \quad \alpha_p^e = 1.00 \quad \alpha_h^e = 1.00$$

Recovery During Acid Hydrolysis Procedure

Monosaccharides. This experiment was performed to evaluate the loss of glucose in the hydrolysis procedure.

Standard glucose solutions containing 100 nmoles of glucose (0.400 ml of 250 μ M) were hydrolyzed for 0 and 3 hours. After hydrolysis the solutions were dried under vacuum at 30°C. The residues found after drying were derivatized in the same manner as standard sugars. The results are given in Table II. There is a 10% loss of glucose just by contact with 3 N HCl. The loss is up to

49% when hydrolysis was extended for three hours. The average percent recovery of glucose vs. the hydrolysis time was plotted for standard dextrin and glucose solutions in Figure 7.

Polysaccharides. Dextrin was used as a polysaccharide and, after hydrolysis, recovery of glucose was obtained. The standard dextrin solution of 250 μ M (glucose equivalent) was prepared. A 0.400 ml sample of the dextrin solution (100 nmoles of glucose) was hydrolyzed with 3 N HCl for 0, 1, 3, 6, and 17 hours. After hydrolysis, the solution was dried and derivatized in the same manner as the glucose solution. The results for recoveries are given in Table II. The maximum yield of glucose from hydrolysis of dextrin was obtained after approximately one hour (Figure 7).

Recovery From XAD-7 Resin

The XAD-7 resin has been used previously to isolate humic substances from natural waters (Mantoura and Riley, 1974). Separation of humic substances from sucrose was obtained by Leenheer and Huffman (1976).

Monosaccharides. The recovery of monosaccharides from XAD-7 resin was obtained for each monosaccharide. For this analysis, 0.4 ml of the standard sugar mixture (250 μ M of each sugar) was acidified to pH 1.6-2.0 by dropwise addition of 0.1 N HCl. This solution contained 100 nmoles of each sugar. The acidified sample was transferred quantitatively to a 10 ml syringe, which was connected to a Swinnex adaptor containing a glass-fiber filter. The sample was filtered directly into a 4 ml XAD-7 column and the syringe was

Figure 7. Percent recovery of glucose vs. hydrolysis time (at 110°C in 3N HCl)

TABLE II
RECOVERY DURING HYDROLYSIS PROCEDURE

Hydrolysis time (hr.)	% Recovery* of glucose	% Recovery of dextrin* (as glucose)
0	{ 90 90	{ 0 0
1		{ 86 88
3	{ 56 42	{ 69 69
6		{ 32 42
17		{ 16 16

*Expected yield of glucose was 100 nmoles.

Humic-Bonded Saccharides. The recovery of humic-bonded saccharides was assumed to be the same as for polysaccharides. It is not possible to obtain percent recovery without knowing the chemical structure of humic-bonded saccharides.

rinsed with 5 ml of 0.01 N HCl into the column. The sample and washings were passed through XAD-7 and the column was washed twice with 5 ml of 0.01 N HCl. All the eluent was collected in a 50 ml beaker, then evaporated to 0.4 ml on a hotplate at 70°C under a stream of filtered air. The concentrated solution was transferred quantitatively into a Reacti-Vial and evaporated again to 0.3 ml. The pH was adjusted to ~8 by adding dropwise 5 N NaOH. The derivatives of these samples were then prepared. The concentration of each sugar was measured in each sample, and the percent recovery was calculated (Table III).

TABLE III
RECOVERY OF MONOSACCHARIDES IN XAD-7 RESIN

<u>% Recovery</u>				
<u>Arabinose</u>	<u>Xylose</u>	<u>Mannose</u>	<u>Galactose</u>	<u>Glucose</u>
99.2	88.0	96.0	90.0	102.0

Expected yield of each sugar was 100 nmoles.

Lytle (1979) has shown that 99% of ^{14}C -glucose is recovered in the eluent which passed through XAD-7 at pH 2. This value agrees well with the results obtained by analysis of alditol acetate derivatives (Table III).

Polysaccharides. Since the fraction of polysaccharides recovered can only be determined by gas chromatography if the solution is hydrolyzed, evaporated, and derivatized, no attempt was made to determine the fraction recovered by gas chromatographic means. The recovery of 75% for ^{14}C -starch obtained by Lytle (1979) was used for the percent recovery of polysaccharides on XAD-7 resin at pH 2.

Humic-Bonded Saccharides. This experiment was performed by absorbance measurements of humic substances which were eluted through XAD-7 at pH 2. The eluted fraction of humic-bonded saccharides was assumed to be equal to the fraction of humic substances which were eluted. This may be an invalid assumption.

One hundred ml of filtered river water from WR-50, WR-56, and 100 ml of 50 mg/l of Williamson River humic substances (collected previously) were acidified to pH 1.6. The XAD-7 columns were

pre-washed with HCl solution (pH 1.6). The samples were passed through the column and each 16 ml fraction was collected in a 17 ml glass bottle containing 0.5 ml of standard pH 10 carbonate buffer. Absorbance was measured for each fraction at 420 nm in 1 cm quartz cells on a Beckman 26 UV-vis Spectrometer. The results of this experiment are given in Table IV.

TABLE IV

ABSORBANCE OF ELUENT VERSUS VOLUME ELUTED

(Absorbance)_{fraction} / (Absorbance)_{initial}

<u>Fraction</u>	<u>WR-50</u>	<u>WR-56</u>	<u>WR-HS*</u>
1 (0-16 ml)	-	-	-
2 (17-32 ml)	0.034	0.000	0.042
3 (33-48 ml)	0.103	0.036	0.014
4 (49-64 ml)	0.103	0.107	0.007
5 (65-80 ml)	0.172	0.107	0.000
6 (81-96 ml)	0.103	0.107	0.00
7 (97-112 ml)	0.103	0.072	0.00
Average [#]	0.103	0.072	0.010

*WR-HS is a 50 mg/l solution of freeze-dried humic substances from the Williamson River.

[#]Fraction 1, which is a pH 1.6 HCl solution is neglected.

The average fraction eluted is 0.088 for the river water samples. This value will be used to estimate the fraction of humic-bonded saccharides recovered in the eluent which passes through XAD-7 resin at pH 2.

Summary Of Fractional Recoveries During Analysis of Sugar Species

The recoveries of each sugar species, using different experimental procedures, are listed in Table V.

TABLE V
FRACTIONAL RECOVERIES FOR SUGAR SPECIES

<u>Procedure</u>	<u>Monosaccharide</u>	<u>Polysaccharide</u>	<u>Humic-Bonded Saccharide</u>
Evaporation	0.950	1.00	1.00
Acid Hydrolysis	0.750	0.870	0.870
XAD-7 Adsorption	0.950	0.750	0.088

If the fraction recovered is α , then for the various steps:

α_m^e = fraction of monosaccharides recovered in the evaporation step

α_p^e = fraction of polysaccharides recovered in the evaporation step

α_h^e = fraction of humic-bonded saccharides recovered in the evaporation step

α_m^a = fraction of monosaccharides recovered in acid hydrolysis

α_p^a = fraction of polysaccharides recovered in acid hydrolysis

α_h^a = fraction of humic-bonded saccharides recovered in acid hydrolysis

α_m^x = fraction of monosaccharides recovered from XAD-7 resin

α_p^x = fraction of polysaccharides recovered from XAD-7 resin

α_h^x = fraction of humic-bonded saccharides recovered from XAD-7 resin

The initial concentrations of monosaccharide (MS), polysaccharide (PS), humic-bonded saccharide (HS), and total saccharide (TS) are defined as: $(MS)_0$, $(PS)_0$, $(HS)_0$, and $(TS)_0$. A modified flow chart

which accounts for fractional recoveries can be constructed for the analysis of the unfractionated water sample (UF) and the fractionated samples (F_1 and F_2). These are shown in Figures 8 and 9.

Based on the fractional recoveries given in Table V, the overall recoveries in the three analytical schemes are:

$$UF = 0.64 (MS)_o + 0.79 (PS)_o + 0.79 (HS)_o \quad (1)$$

$$F_1 = 0.90 (MS)_o \quad (2)$$

$$F_2 = 0.64 (MS)_o + 0.62 (PS)_o + 0.073 (HS)_o \quad (3)$$

By simultaneous solution of these three equations (1, 2, 3), the initial concentrations of monosaccharide $(MS)_o$, polysaccharide $(PS)_o$ and humic-bonded saccharide $(HS)_o$ can be calculated. The final equations used are:

$$(MS)_o = 1.111 F_1 \quad (4)$$

$$(PS)_o = 1.828 F_2 - 1.180 F_1 - 0.169 UF \quad (5)$$

$$(HS)_o = 1.435 UF + 0.265 F_1 - 1.828 F_2 \quad (6)$$

$$(TS)_o = 1.266 UF + 0.211 F_1 \quad (7)$$

It should be noted that the results of the fractionation scheme are required for an unambiguous calculation of $(TS)_o$. However, because the monosaccharide fraction was found to be consistently small (see Results), the total dissolved sugar in the unfractionated samples (e.g., January and February water samples) was estimated from the analysis of the unfractionated sample using Eq. 7 and assuming that $(MS)_o = 0$. The resulting equation is:

$$(TS)_o = 1.266 UF \quad (8)$$

which will yield slightly low results due to the exclusion of monosaccharides in the modified equation.

Figure 8. Overall recovery of sugars in unfractionated samples.
(See Table V for percent recoveries in each step.)

Figure 9. Overall recovery of sugars in fractionated samples.
(See Table V for percent recoveries in each step.)

Because of the very low concentrations of some sugar species, the calculated concentrations of some species from Eq. 4-7 are occasionally less than zero. In these instances, the concentration of that species is assumed to be zero and the remaining species are decreased proportionally until the total dissolved sugar concentration equals $(TS)_0$ as calculated from Eq. 8.

COLLECTION OF WATER SAMPLES

Samples were collected in January, February and March, 1979 in one liter linear polyethylene bottles at each sample site. Two ml of 1.5 M NaN_3 solution was added to each bottle as a preservative. All samples were stored under refrigeration at 5°C before analysis.

Other samples were taken for analyses of major ions and algae. Temperature, pH, dissolved oxygen, and discharge were measured in the field at each sample site (the results of these analyses will be published elsewhere).

RESULTS

The first attempt was to make trimethylsilyl ether derivatives of the sugars, but, due to the presence of open-chain, α -pyranose, β -pyranose, and other forms, each sugar yielded a complicated chromatogram which was difficult to analyze quantitatively. Alditol acetate derivatives of sugars did not have those problems. Each sugar had only one derivative, the derivatization procedure was very reproducible, and the derivatives were stable over two months in capped vials. Typical chromatograms for a standard and a water sample are given in Figure 10.

The total dissolved sugars from unfractionated samples were measured for January, February and March, 1979. The results are in Tables VI, VII, and VIII.

The fractionated analysis was obtained for WR-10, WR-50 and WR-56 in March, 1979. The initial concentrations of sugar species (MS, PS and HS) in WR-10, WR-50, and WR-56 were calculated by using equations 4-7 for the March, 1979 samples. These results are given in Table IX.

Figure 10. Comparison of chromatograms of a standard sugar solution and a river sample.

TABLE VI

CONCENTRATIONS OF SUGARS (μ M) FROM WATER SAMPLES IN THE
WILLIAMSON RIVER SYSTEM IN JANUARY, 1979

<u>Sample</u>	<u>Arabinose</u>	<u>Xylose</u>	<u>Mannose</u>	<u>Galactose</u>	<u>Glucose</u>	<u>Total</u>
WR-10	0.03 (0.00)**	0.01 (0.00)	0.02 (0.00)	0.04 (0.01)	0.02 (0.00)	0.13 (0.01)
WR-21	0.51 (0.13)	0.56 (0.04)	0.33 (0.03)	0.46 (0.01)	0.56 (0.09)	2.41 (0.25)
WR-30	0.89 (0.03)	0.99 (0.01)	0.63 (0.07)	0.86 (0.09)	1.24 (0.03)	4.61 (1.33)
WR-32	1.47 (0.28)	2.15 (0.86)	0.66 (0.07)	0.94 (0.16)	0.96 (0.24)	6.18 (0.14)
WR-50	1.87 (0.03)	2.38 (0.17)	0.61 (0.06)	1.39 (0.16)	1.42 (0.13)	7.67 (0.20)
WR-56	0.89 (0.02)	1.39 (0.02)	0.63 (0.06)	1.14 (0.11)	1.29 (0.13)	5.34 (0.34)
SC-20	0.03 (*)	0.00 (*)	0.03 (*)	0.05 (*)	0.09 (*)	0.20 (*)
WR-67	0.35 (0.01)	0.58 (0.06)	0.25 (0.06)	0.48 (0.00)	0.46 (0.07)	2.13 (0.04)
SR-65	0.58 (0.25)	0.51 (0.09)	0.35 (0.16)	0.56 (0.08)	0.68 (0.20)	2.68 (0.64)
WR-80	0.38 (0.05)	0.46 (0.05)	0.33 (0.02)	0.46 (0.05)	0.46 (0.03)	2.08 (0.17)
KL-10	0.30 (0.07)	0.43 (0.11)	0.33 (0.10)	0.58 (0.07)	0.78 (0.12)	2.43 (0.37)

**Average deviation of duplicate analyses.

*Only one value obtained.

TABLE VII

CONCENTRATIONS OF SUGARS (μM) FROM WATER SAMPLES IN THE
WILLIAMSON RIVER SYSTEM IN FEBRUARY, 1979 (SINGLE ANALYSIS)

<u>Sample</u>	<u>Arabinose</u>	<u>Xylose</u>	<u>Mannose</u>	<u>Galactose</u>	<u>Glucose</u>	<u>Total</u>
WR-10	0.01	0.00	0.00	0.07	0.00	0.08
WR-21	0.07	0.05	0.06	0.00	0.04	0.22
WR-30	0.46	0.01	0.04	0.51	0.05	1.06
WR-32	1.52	1.56	0.49	2.30	0.76	6.63
WR-50	1.79	1.75	0.41	2.55	0.96	7.45
WR-56	0.78	0.71	0.26	0.78	0.47	2.99
SC-20	0.05	0.00	0.00	0.01	0.05	0.11
WR-67	0.44	0.24	0.08	0.70	0.26	1.72
SR-65	1.39	1.90	0.58	1.63	1.09	6.59
WR-80	1.13	0.97	0.32	1.01	0.57	4.00
KL-10	0.27	0.24	0.29	0.41	0.43	1.63

TABLE VIII
CONCENTRATIONS OF SUGARS (μ M) FROM WATER SAMPLES IN THE
WILLIAMSON RIVER SYSTEM IN MARCH, 1979

<u>Sample</u>	<u>Arabinose</u>	<u>Xylose</u>	<u>Mannose</u>	<u>Galactose</u>	<u>Glucose</u>	<u>Total</u>
WR-10	0.09 (*)	0.07 (*)	0.12 (*)	0.03 (*)	0.00 (*)	0.41 (*)
WR-21	0.12 (0.00)**	0.09 (0.04)	0.13 (0.01)	0.23 (0.03)	0.17 (0.04)	0.74 (0.12)
WR-30	0.08 (0.20)	0.08 (0.20)	0.05 (0.04)	0.01 (0.00)	0.07 (0.01)	0.29 (0.08)
WR-32	0.60 (0.15)	0.75 (0.15)	0.28 (0.03)	1.25 (0.14)	0.63 (0.04)	3.52 (0.52)
WR-50	1.12 (0.01)	1.55 (0.17)	0.61 (0.13)	1.85 (0.10)	1.26 (0.24)	6.39 (0.65)
WR-56	1.14 (0.04)	0.96 (0.13)	0.83 (0.10)	0.94 (0.06)	0.67 (0.03)	4.54 (0.52)
SC-20	0.04 (*)	0.01 (*)	0.05 (*)	0.02 (*)	0.02 (*)	0.13 (*)
WR-67	0.44 (0.04)	0.37 (0.04)	0.19 (0.01)	0.20 (0.12)	0.30 (0.04)	1.51 (0.08)
SR-65	0.75 (0.09)	0.58 (0.07)	0.54 (0.03)	0.52 (0.01)	0.59 (0.03)	2.98 (0.21)
WR-80	0.36 (0.02)	0.51 (0.05)	0.23 (0.05)	0.38 (0.09)	0.33 (0.02)	1.81 (0.18)
KL-10	0.22 (0.00)	0.38 (0.01)	0.29 (0.00)	0.58 (0.00)	0.33 (0.00)	1.80 (0.02)

**Average deviation of duplicate analyses.

* Only one value obtained.

TABLE IX

CONCENTRATION OF FRACTIONATED SUGARS (μM) FROM WATER SAMPLES IN THE
WILLIAMSON RIVER SYSTEM IN MARCH, 1979 (SINGLE ANALYSIS)

<u>Sample</u>	<u>Arabinose</u>	<u>Xylose</u>	<u>Mannose</u>	<u>Galactose</u>	<u>Glucose</u>	<u>Total</u>
WR-10 (MS) _o	0.03	0.01	0.00	0.01	0.01	0.06
WR-10 (PS) _o	0.01	0.00	0.00	0.00	0.00	0.01
WR-10 (HS) _o	0.05	0.06	0.12	0.02	0.09	0.34
WR-10 (TS) _o	0.09	0.07	0.12	0.03	0.10	0.41
WR-50 (MS) _o	0.07	0.01	0.00	0.02	0.03	0.13
WR-50 (PS) _o	0.42	0.40	0.61	0.90	0.68	3.01
WR-50 (HS) _o	0.64	1.15	0.00	0.93	0.55	3.27
WR-50 (TS) _o	1.13	1.56	0.61	1.85	1.26	6.41
WR-56 (MS) _o	0.06	0.02	0.00	0.01	0.02	0.11
WR-56 (PS) _o	0.00	0.00	0.16	0.16	0.29	0.61
WR-56 (HS) _o	1.09	0.94	0.67	0.76	0.37	3.83
WR-56 (TS) _o	1.15	0.96	0.83	0.93	0.68	4.55

DISCUSSION

Gas chromatographic analysis of alditol acetate derivatives of dissolved carbohydrates in the Williamson River was a sensitive method for the determination of individual sugars, with a detection limit of 25 nM. The detection limit obtained with the paper chromatographic method of Whittaker and Vallentyne (1957) was 1 μ g per spot for each sugar. With the gas chromatographic method used in this study, the detection limit per injection is 25 pmoles, or 4.50 ng of each hexose and 3.75 ng of each pentose. The sensitivity obtained by the gas chromatographic method in this study is approximately 200 times greater than with the paper chromatographic method.

Because of the very low detection limit, only 100 ml of each sample were used in this study. Vallentyne and Whittaker (1956) needed 2,000 - 5,000 ml, and Degens et al. (1964) required 2,000 ml. The total time for preparation of the samples prior to derivatization, for derivatization, and for gas chromatographic analysis was approximately 24 hours for all 11 samples, including a 10 hour evaporation time. This is not very long in comparison with three days required for paper chromatography.

In this study, the losses of sugars in each step were determined and the overall recovery of 79% at low concentrations were obtained. The ion exchange resin which was used by many investigators (Vallentyne and Bidwell, 1956; Vallentyne and Whittaker, 1956; and Whittaker and

Vallentyne, 1957) reportedly caused losses of about 25% of the sugars. This kind of resin was not used in this research, and inorganic salt residues did not interfere with the sugar analysis.

Total dissolved sugar concentrations were 0.08-7.67 μM for the Williamson River system. Bikbulatov and Skopintsev (1974) found from 10 to 22 μM dissolved sugars in natural waters containing high concentrations of humic substances. Walsh (1965) obtained dissolved sugar concentrations in the range of 6 - 18 μM . The sugar carbon in the Williamson River constitutes a very small fraction of the total organic carbon (TOC), being only 0.2 to 2.0% of TOC. Birge and Juday (1934) estimated that carbohydrate constituents are 73.4 - 89.9% of the total dissolved organic matter in many Wisconsin lakes. Obviously these data are much higher than the data reported by other investigators and the data obtained in this study.

The results in Tables VI - VIII and Figures 11 - 13 graphically illustrate how the individual and total sugar concentrations vary throughout the Williamson River system during each month.

The spring waters (WR-10 and SC-20) generally contain very few algae, and also have low TOC's. These samples are also quite low in dissolved sugars. As the Williamson River enters Klamath Marsh, the sugar concentration, TOC, and color (as absorbance at 420 nm) all increase substantially. The sugar concentration reaches a maximum at WR-50, and, after that point in the river, dilution from small springs and tributaries reduces the concentration of the sugars.

It can be seen from Figures 11, 12 and 13 that about 50% of the total dissolved sugars are pentoses, with xylose being generally

Figure 11. The distribution of concentrations of individual sugars for January, 1979.

*See Table VI for individual sugar concentrations.

Figure 12. The distribution of concentrations of individual sugars for February, 1979.

*See Table VII for individual sugar concentrations.

Figure 13. The distribution of concentrations of individual sugars for March, 1979.

*See Table VIII for individual sugar concentrations.

more abundant than arabinose. Among the hexoses, mannose is present at a low but relatively constant concentration. Galactose and glucose are very nearly equal throughout most of the Williamson River system.

The results in Table IX show that monosaccharides account for 2-14% of the total sugar concentrations for those sample sites for which the fractionation procedure was used. Based on these limited results, it appears that monosaccharides represent a larger fraction of total saccharides in spring water than in waters which are high in humic substances. Nevertheless, the actual monosaccharide concentrations were similar in both types of waters.

Because of the small fraction of humic substances which is not adsorbed on XAD-7 resin, there is some analytical difficulty in distinguishing between polysaccharides and humic bound saccharides. On the basis of the fractionation that has been done, it seems that the polysaccharide fractions are more variable than the monosaccharides. In all three samples, half or more of the dissolved sugars appear to be associated with humic substances.

To gain more insight into the factors that may tend to affect the sugar content in the Williamson River, statistical correlations between the total dissolved sugar concentrations and other potentially related variables were calculated (Table X). These results indicate a strong correlation between total dissolved sugars and absorbance at 420 nm (a measure of humic substances - see Figure 14) but a weaker correlation with TOC. The statistical results support the results of the fractionation study, which showed that the majority of sugars were associated with the humic substances. A good correlation

TABLE X
CORRELATION COEFFICIENTS FOR TOTAL DISSOLVED
SUGARS VERSUS POTENTIALLY RELATED VARIABLES #

<u>Sample date</u>	<u>TOC</u>	<u>Absorbance</u>	<u>Amino acids</u>	<u>Silica</u>	<u>Nitrate</u>	<u>Phosphate</u>
Jan. 1979	0.744**	0.893**	0.859**	-0.683*	-0.238	-0.418
Feb. 1979	0.569	0.797**	0.678	-0.743**	-0.655*	-0.193
Mar. 1979	0.404	0.885**	0.736**	-0.915**	-0.405	-0.578

*significant correlation at the 95% confidence level

**significant correlation at the 99% confidence level

#See Appendix C

Figure 14. Total sugar concentrations (μM) versus absorbance (at 420 nm).

was also found between total dissolved sugars and amino acids. Other research which is currently underway in this laboratory has shown that most amino acids are bonded to humic substances (Lytle, 1979).

There is a strong negative statistical correlation between total dissolved sugar concentrations and silica concentration (Table X and Figure 15), which may indicate that the sugar concentration in the water is related to the growth of diatoms. There is not a good correlation between total dissolved sugars and nitrate or phosphate concentrations, which are those species which usually limit the algal productivity.

Figure 15. Total sugar concentrations (μM) versus silica concentrations (μM).

CONCLUSIONS

Alditol acetate derivatives of sugars are suitable for gas chromatographic analysis of carbohydrates in natural waters. Only one chromatographic peak was obtained for each sugar and the total time required for elution is twenty minutes. The total time required to prepare twelve water samples for analysis is approximately twenty-four hours. The detection limit is 25 nM for each sugar. The volume of water sample is 100 ml. Despite the small volume of samples, overall recovery (approximately 79%) is better than for other methods. With the use of XAD-7 to selectively adsorb humic substances, additional information about the concentrations of dissolved sugars in the form of monosaccharides, polysaccharides, and humic-bonded saccharides is obtainable.

The concentrations of hydrolyzable carbohydrates were obtained for eleven stations of the Williamson River system in January, February, and March, 1979. Each month, individual sugars (arabinose, xylose, mannose, galactose, and glucose) ranged from 0.00 to 2.55 μM , with total sugars ranging from 0.08 to 7.67 μM . The highest concentration of sugars appeared in those sample sites with highest concentrations of humic substances. After flowing through Klamath Marsh, the total concentration of sugars increased by a factor of about 5. In contrast, the total concentration of sugars in spring waters ranged from 0.08 to 0.20 μM .

The fractionation analysis of dissolved monosaccharides, polysaccharides, and humic-bonded saccharides, which was performed for three samples in March, 1979, gave very low concentrations of monosaccharides, which accounted for about 10% of the total sugar in spring waters and about 2.5% of the total sugars in humic-rich waters. Polysaccharides were highly variable, accounting for 1-50% of the total sugars. The remaining sugars are apparently strongly adsorbed or covalently bonded to humic substances.

SUMMARY

Gas chromatographic analyses of alditol acetate derivatives of pentoses (arabinose and xylose) and hexoses (mannose, galactose, and glucose) were investigated for determination of dissolved sugar concentrations in the Williamson River system. Dissolved sugar concentrations were measured for all samples in January, February, and March of 1979.

Dissolved sugar concentrations ranged from 0.08 to 7.67 μM , with the higher concentrations occurring in the humic-rich samples, and the lower concentrations in spring waters. Pentoses were about 50% of the total sugars.

The fractionation analyses were performed for determination of dissolved free monosaccharides, polysaccharides, and humic-bonded saccharides for three sample sites in March, 1979. Approximately 91% of the humic substances could be removed by adsorption on XAD-7 resin. The concentrations found were in the range of 0.00 - 0.13 μM of monosaccharides, 0.00 - 3.01 μM of polysaccharides, and 0.34 - 3.83 μM of humic-bonded saccharides. The concentrations of monosaccharides were very low in all of the samples. More than 50% of the sugars were bound to humic substances.

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APPENDIX A

LIST OF CHEMICALS

<u>Name of Chemicals</u>	<u>Company</u>	<u>Location</u>
Acetic acid	Mallinckrodt Chemical Works	St. Louis, MO 63160
Acetic anhydride (analytical reagent)	Mallinckrodt Chemical Works	St. Louis, MO 63160
Chloroform	J.T. Baker Chemical Co.	Phillipsburg, NY 08865
Ethanol (absolute)	Imc. Chemical Group, Inc.	Agnew, CA
Hydrochloric acid	J.T. Baker Chemical Co.	Phillipsburg, NY 08865
Inositol acetate		
Methanol (absolute)	J.T. Baker Chemical Co.	Phillipsburg, NY 08865
Pyridine	Mallinckrodt Chemical Works	St. Louis, MO 63160
Sodium azide	Mallinckrodt Chemical Works	St. Louis, MO 63160
Sodium Borohydride	Eastman Kodak Co.	Rochester, NY 14650
Arabinose Xylose Sugars	Aldrich Chemical, Inc.	Milwaukee, WI 53233
Mannose Galactose } Glucose	Matheson, Coleman and Bell	Cincinnati, OH

APPENDIX B

CHEMICAL STRUCTURES AND FORMULAS

Hexoses:

D-glucose

D-galactose

D-mannose

Pentoses:

D-arabinose

D-xylose

In a solution, the pyranose forms predominate, e.g., the glucose pyranose forms are:

β -D-glucopyranose

α -D-glucopyranose

Alditol and Alditol Acetates

An alditol is a polyhydroxyl compound, which is obtained by reduction of the aldehyde group of the sugar to the hydroxyl group.

The alditol of glucose is:

The alditol acetate of a sugar is obtained by reacting the alditol of the sugar with acetic anhydride. For example, the alditol acetate of glucose is: