# PAPER CHROMATOGRAPHY OF MONOMER SUGARS USING QUATERNARY SALTS FOR IDENTIFICATION

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## INTRODUCTION

A number of procedures utilizing aniline oxalate[1], cysteine–carbazole[2], 2,4-dinitrophenylhydrazine[3], resorcinol[4], naphthoresorcinol[5], orcinol[6], vanillin[7], *p*-anisidine[1], urea hydrochloride[1], *o*-phenylenediamine[8], diphenylamine–aniline[9], Benedict's solution[10], ammoniacal silver nitrate[11] have been used for the detection of sugars on paper chromatograms. The procedures yield color differences and are sensitive to ketoses and aldoses. However, uronic acids, 5-hexulosonic acids and sugar amines fail to be selectively differentiated by the above methods.

CHARGAFF *et al.*[12] used a *m*-phenylenediamine solution and found that complexes with unsubstituted sugars possessed a marked fluorescence in the ultra-violet. ROREM[13] has been successful in detecting and differentiating ketoses and aldoses with the aid of ultra-violet fluorescence of sulfosalicylic acid. ROREM[14] was able to detect phosphorylated sugars as well as amino acids by a fluorescence technique using quinine sulfate. BERA *et al.*[15] have also reported the use of cetyl-trimethyl-ammonium bromide (Cetavlon) as a spray reagent for the identification of sugars.

A major phase of our work is related to the identification of degradation products from acid mucopolysaccharides such as heparin, heparinoids, chondroitin sulfates and hyaluronic acid. A need developed during the course of our investigation for a chromagen which would permit identification of different monomer sugar classes, and subsequently different polymer fragments. This paper reports the use of organic reagents containing nitrogen which are substituted with alkyl or aryl groups capable of producing quaternary ammonium ions as possible identifying reagents. In this study, the ultra-violet fluorescence or, quenching, of the quaternary ammonium ion complex was used to detect and differentiate monomer sugars.

## EXPERIMENTAL

### *Sample materials*

The sugars or sugar derivatives used in this investigation were either obtained commercially or prepared in this laboratory. All of the samples were dissolved in water and were made up to a concentration of 1% (w/v). Multiples of one microliter aliquots were applied to the paper with a micro pipette in order to assess the sensitivity of the chromagen under investigation.

* Deceased.

*Developing solvents*

The following solvent systems were used:

(A) Pyridine–ethyl acetate–acetic acid–water (5:5:1:3)[16]
(B) Butanol–ethanol–water (4:1.1:1.9)
(C) *tert.*-Amyl alcohol–propanol–ethanol–water (4:1.3:0.5:2)
(D) Butanol–acetic acid–water (5:1.5:3.5)[17]
(E) Butanol–acetic acid–water (5:1.4:2.9)[17]
(F) Butanol–acetic acid–water (5:1.2:2.5)[17]
(G) *tert.*-Amyl alcohol–formic acid–water (4:1:1.5)
(H) Butanol–acetic acid–water (4.4:1.6:4.0)[17]

The above solvent systems are miscible at room temperature. In all cases descending chromatography was used.

*Paper*

Whatman No. 1 paper was found to be suitable for the procedure. Prior washing of the paper with the solvent in question was found to be beneficial, but not essential in qualitative work. Papers washed with water had little advantage over unwashed papers.

*Detection*

The following quaternary ammonium salts were used: (1) quinacrine hydrochloride, (2) Pyronin Y, (3) proflavine hydrochloride, (4) sparine hydrochloride, (5) methyl-dodecylbenzyl-trimethyl-ammonium chloride (Hyamine 1622), (6) cetyl-trimethyl-ammonium bromide (Cetavlon), (7) cetylpyridinium chloride, (8) cetyl-dimethyl-benzyl-ammonium chloride. Each of the reagents was prepared in stock solutions containing 500 mg in 200 ml of 80% ethanol. The stock solution was diluted 1:10 with 80% ethanol for spray application.

A long wave lamp with ultra-violet radiation in the 3600Å range was used for the identification of the quaternary complexes of the sugar samples.

These lights are constructed of a special high-transmitting self-filtering glass and hence require no secondary filter. At this wavelength, the sprayed chromatographic paper shows a faint fluorescence, and the substances under analysis appear as areas of quenching with slight coloration.

*Procedure*

After the chromatogram has been developed, the respective solvent is removed by first air drying at room temperature, followed by drying at 100° for 3–5 min. The paper is sprayed with the desired quaternary reagent. The sprayed paper is allowed to dry at room temperature under forced air conditions. The sprayed paper is then heated for 3–5 min at 100°.

Under ultra-violet light one can locate and identify the areas of quenching.

## RESULTS AND DISCUSSION

The initial phase of this investigation (*cf.* Table I) relates the sensitivity of the respective quaternary compounds to the monomer sugars. From previous work[17] the

TABLE I

QUATERNARY EVALUATION

Solvent system (H): butanol–acetic acid–water (4.4:1.6:4.0)

| *Quaternary salt* | | *1* | *2* | *3* | *4* | *5* | *6* | *7* | *8* |
|---|---|---|---|---|---|---|---|---|---|
| *sensitivity class* | | *2* | *3* | *1* | *3* | *3* | *3* | *3* | *2* |
| Glucose | $R_F$ | 0.47 | | 0.46 | | | 0.50 | | 0.45 |
| | Color | green | none | green | none | none | white | none | white |
| Mannose | $R_F$ | 0.49 | | 0.49 | | | 0.53 | | |
| | Color | lt. green | none | green | none | none | white | none | none |
| 2,5-Anhydro-mannose | $R_F$ | 0.80 | | 0.85 | | | 0.80 | | |
| | Color | violet | none | blue | none | none | white | none | none |
| Glucurone | $R_F$ | 0.49 | | 0.53 | 0.53 | 0.55 | 0.55 | 0.54 | 0.54 |
| | Color | yellow | none | orange | blue | white | white | white | white |
| Glucuronic acid | $R_F$ | 0.43 | | 0.41 | 0.45 | 0.48 | 0.45 | 0.44 | 0.43 |
| | Color | lt. green | none | orange | blue | white | white | white | white |
| Galacturonic acid | $R_F$ | 0.41 | | 0.36 | 0.38 | 0.42 | | 0.40 | 0.40 |
| | Color | lt. green | none | orange | violet | white | none | white | white |
| Brucine salt of D-lyxo-5-hexulosonic acid | $R_F$ | 0.78 | 0.83 | 0.82 | 0.83 | 0.76 | 0.79 | 0.79 | 0.76 |
| | Color | violet | violet | blue | violet | violet | violet | white | violet |
| Brucine salt of D-xylo-5-hexulosonic acid | $R_F$ | 0.79 | 0.82 | 0.82 | 0.83 | 0.77 | 0.79 | 0.80 | 0.77 |
| | Color | violet | violet | blue | violet | violet | violet | white | violet |
| Galactosamine | $R_F$ | 0.32 | 0.32 | 0.33 | 0.31 | 0.37 | | 0.36 | 0.35 |
| | Color | lt. green | violet | violet | blue | white | none | white | white |
| Glucosamine | $R_F$ | 0.33 | 0.35 | 0.34 | 0.35 | 0.37 | | 0.39 | 0.44 |
| | Color | violet | violet | violet | white | white | none | white | white |

butanol–acetic acid–water solvent system (H) was selected for the initial phase. The following classification of sensitivity was chosen and was used to select the most appropriate quaternary compound.

Class 1: 10–50 $\mu$g of sugar produces visually detectable level of color,

Class 2: 50–100 $\mu$g of sugar produces visually detectable level of color,

Class 3: greater than 100 $\mu$g of sugar produces visually detectable level of color.

Sugars are reported in terms of both $R_F$ values as well as the color of the complex under ultra-violet light.

It has been demonstrated from this work that proflavin hydrochloride is the most sensitive of the reagents examined. With solvent system (H) and proflavin hydrochloride (3), the hexoses appear as green spots; the amines appear as violet spots; whereas the uronic acids and lactones appear as orange spots. The brucine salts of the 5-hexulosonic acids, however, appear as blue spots.

Table II shows the influence of solvents upon the color of the quaternary complex when viewed under ultra-violet light using proflavin hydrochloride as the identifying reagent. It should be noted that galacturonic acid, galactosamine, glucosamine and 2,5-anhydromannose respectively exhibit different colors with different solvents. It is therefore possible to resolve a mixture containing a uronic acid, a 5-hexulosonic acid and a sugar amine and a hexose. As an example, (*cf.* Table II) this could be accomplished with glucosamine, galacturonic acid, brucine salt of D-lyxo-5-hexulosonic

TABLE II

SOLVENT EVALUATION

Chromagen (3): proflavin hydrochloride (18 h development time)

| *Solvent* | | *A* | *B* | *C* | *D* | *E* | *F* | *G* | *H* |
|---|---|---|---|---|---|---|---|---|---|
| Glucose | $R_F$ | 0.33 | 0.21 | 0.19 | 0.31 | 0.23 | 0.24 | 0.19 | 0.46 |
| | Color | green | green | green | green | green | green | green | green |
| Mannose | $R_F$ | 0.34 | 0.28 | 0.29 | 0.39 | 0.30 | 0.30 | 0.24 | 0.49 |
| | Color | green | green | green | green | green | green | green | green |
| 2,5-Anhydro-mannose | $R_F$ | 0.70 | 0.16 | 0.20 | 0.08 | 0.43 | 0.24 | 0.37 | 0.85 |
| | Color | orange | violet | violet | violet | violet | violet | orange | violet |
| Glucurone | $R_F$ | 0.77 | 0.38 | 0.42 | 0.45 | 0.40 | 0.40 | 0.35 | 0.53 |
| | Color | orange | orange | orange | orange | orange | orange | orange | orange |
| Glucuronic acid | $R_F$ | 0.32 | 0.24 | 0.25 | 0.30 | 0.22 | 0.21 | 0.18 | 0.41 |
| | Color | orange | orange | orange | orange | orange | orange | orange | orange |
| Galacturonic acid | $R_F$ | 0.16 | 0.22 | 0.21 | 0.24 | 0.18 | 0.12 | 0.11 | 0.36 |
| | Color | orange | orange | orange | orange | violet | violet | violet | orange |
| Brucine salt of D-lyxo-5-hexulosonic acid | $R_F$ | 0.80 | 0.48 | 0.31 | 0.75 | 0.65 | 0.67 | 0.62 | 0.82 |
| | Color | blue | blue | blue | blue | blue | blue | blue | blue |
| Brucine salt of D-xylo-5-hexulosonic acid | $R_F$ | 0.80 | 0.45 | 0.29 | 0.74 | 0.63 | 0.66 | 0.61 | 0.82 |
| | Color | blue | blue | blue | blue | blue | blue | blue | blue |
| Galactosamine | $R_F$ | 0.26 | 0.06 | 0.18 | 0.22 | 0.08 | 0.11 | 0.09 | 0.33 |
| | Color | green | green | violet | violet | violet | violet | violet | violet |
| Glucosamine | $R_F$ | 0.18 | 0.16 | 0.12 | 0.22 | 0.18 | 0.14 | 0.16 | 0.34 |
| | Color | green | green | green | green | green | violet | green | violet |

acid and glucosamine by first using solvent system (A) and chromagen (3), followed by a duplicate set of chromatograms using solvent system (E) and chromagen (3). An alternative would be to use solvent system (A) followed by solvent system (D) and again using chromagen (3) for both.

SUMMARY

A selective procedure has been developed for the identification of hexoses, sugar acids and lactones, sugar amines and 5-hexulosonic acid salts with the aid of a quaternary ammonium compound. When proflavin hydrochloride is complexed with the respective sugars, viewed under ultra-violet light, the monomers are identified by various $R_F$ values and colors. The procedure is sensitive to 10 micrograms of sugar.

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