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A NEW SENSITIVE ULTRAVIOLET DETECTION SYSTEM FOR CARBOHYDRATES ELUTED DURING COLUMN CHROMATOGRAPHY*

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SUMMARY

A detection system for carbohydrates eluted from chromatographic columns has been developed that is simple and sensitive. It relies upon the production of an ultraviolet-absorbing chromophore by reaction of the carbohydrate in the eluent solution dynamically with sulfuric acid.

INTRODUCTION

A simple and sensitive system for continuously monitoring carbohydrates and related compounds as they elute from chromatographic columns has been developed. This system depends upon the rapid production of chromophores with absorption maxima in the 290–310 nm region by dynamic mixing of the column eluent with sulfuric acid. This new system overcomes the reagent instability and recalibration problems associated with anthrone[1], orcinol[2], and similar reagents; the peak broadening and base line noise associated with the sequential addition of two reagents with phenol[3]; and the peak broadening associated with the long reaction time with aniline[4].

The reaction of carbohydrates with sulfuric acid to produce UV-absorbing chromophores has been extensively studied. In the earliest work[5–11] the conversion of D-glucose and other hexoses to 5-(hydroxymethyl)-2-furaldehyde on heating with acids was explored, and the absorption maximum for the chromophore was found to be near 300 nm. The pentoses[12–16] were similarly treated to produce chromophores with absorption maxima near 320 nm and molar absorption coefficients of 10 000–20 000. More recently, similar reactions with sulfuric acid have been reported for 2-deoxyribose[17]; deoxyribonucleic acid[18]; ribonucleic acid[19]; hydroxylated hexoses and pentoses, ketoses, 2-deoxyhexoses, methylpentoses, and streptomycin (which contains a deoxypentose)[20,21]; and uronic acid[21,22]. Studies have generally involved reaction periods of hours, under a variety of temperature and acid conditions.

Our goal was to determine a single set of operating parameters for carrying out the carbohydrate–sulfuric acid reaction dynamically and reproducibly in a few

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minutes with good sensitivity for the large variety of carbohydrates and related compounds found in body fluids.

EXPERIMENTAL

This study had three parts: the determination of optimum reaction conditions, the design of a suitable flow photometer, and the testing of the monitoring system as a part of a working carbohydrate analyzer.

Optimum reaction conditions

The experimental parameters—the reactor temperature and the flow rate of sulfuric acid—were varied using a mixer-reactor of the type used in the Mark III carbohydrate analyzer[23]. The simulated eluent flow was 11 ml/h of a solution containing 0.01 μmole/ml of each of the following sixteen sugars: deoxyribose, sucrose, raffinose,

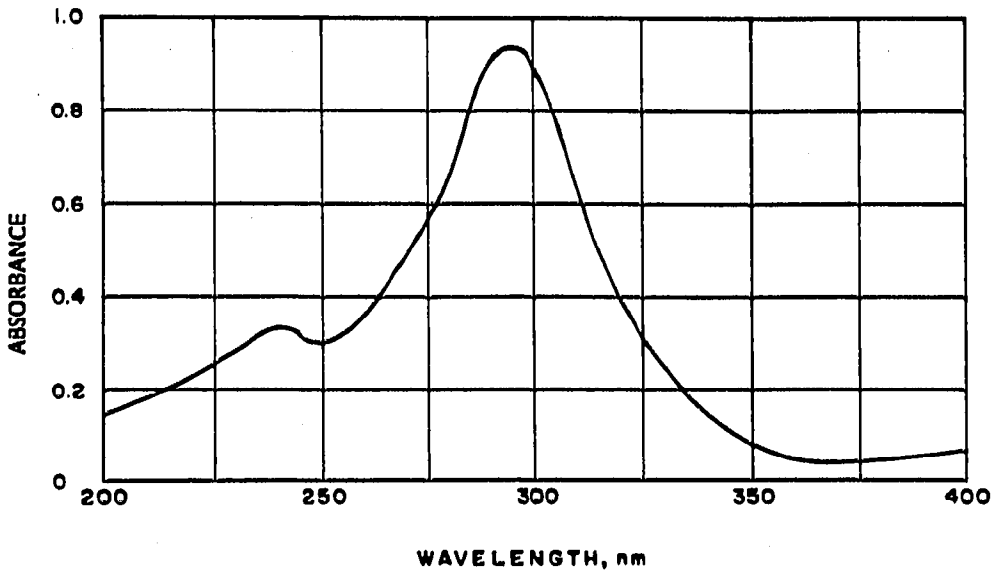

Fig. 1. Absorption spectrum of the product of dynamic mixing of 11 μl/h of an equimolar solution of 16 sugars (0.01 μmole/ml of each) and 19 ml/h of sulfuric acid through 110° reactor for *ca.* 2 min.

cellobiose, maltose, lactose, rhamnose, ribose, mannose, fructose, arabinose, galactose xylose, sorbose, glucose, and melibiose. The eluent flow was fixed at 10–12 ml/h to make the monitor compatible with the Mark III carbohydrate analyzer[23], and the mixer-reactor design used in that analyzer was adopted because of the favorable experience with its use there.

For each set of reactor temperature and sulfuric-acid-flow conditions the product was collected and the absorbance measured in the 240–400 nm region. The position, size, and number of absorption peaks were found to be related to the operating parameters. The operating conditions were adjusted to give a single largest absorption peak at 296 nm and a minimum near 254 nm, as shown in Fig. 1; the reactor temperature was near 110°, and the sulfuric acid flow was 19 ml/h. Variations of ±10° in temperature and ±2 ml/h in flow resulted in little change in the spectrum.

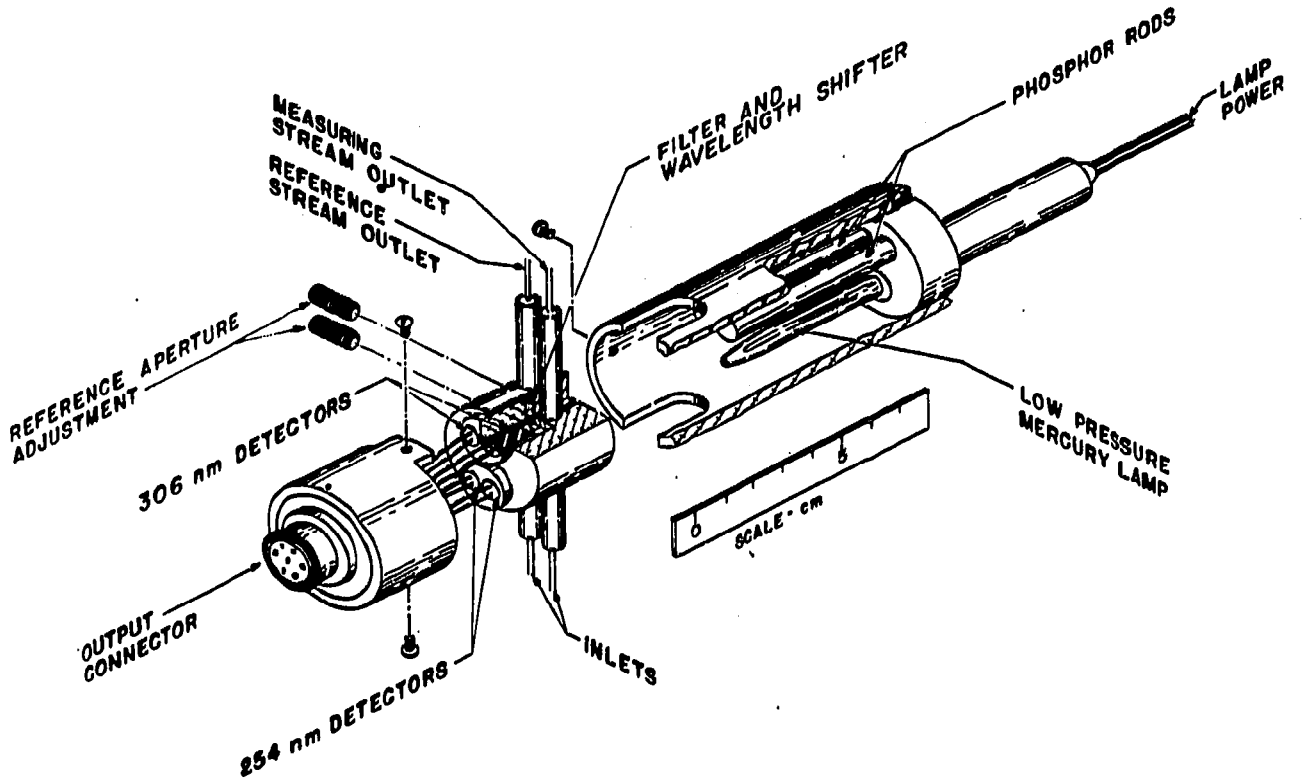

Fig. 2. Two-wavelength UV photometer detector head.

Flow photometer

Because of its sensitivity, simplicity, and operational durability, a modification of the previously developed miniature dual-beam photometer with two fixed wavelengths was adopted[24,25]. The 254-nm channel was retained, because of the minimum absorption of carbohydrates at that wavelength, to help identify non-carbohydrate compounds. To provide better discrimination from other constituents of body fluids which absorb strongly below 300 nm, an analytical channel at 306 nm rather than at the 296-nm optimum of Fig. 1 was substituted for the normal 280-nm channel. The choice of this wavelangth was due to the availability of a suitable phosphor.

The analytical channel was modified by placing between the flow cell and the photodetector a filter and wavelength shifter consisting of a Corning 7-54 filter and a cover glass with an interior layer of Sylvania phosphor type 2382. Therefore the analytical channel of the modified photometer (Fig. 2) consists of a phosphor-rod light source (LKB Instrument Inc. Part No. 8390-02), a quartz flow cell with a 0.27-cm optical path, a Corning 7-54 filter, a Sylvania phosphor type 2382, and a Clairex type Cl 907HL photoconductor. The phosphor rod is activated by the 254-nm light of the mercury lamp and emits the broad light spectrum shown in Fig. 3. The UV portion of that light passes through the Corning filter and is absorbed by the Sylvania phosphor with the efficiency also shown in the figure, giving the overall peak sensitivity at 306 nm. The Sylvania phosphor emits proportional light at 620 nm, the optimum region of the photoconductor sensitivity.

Testing of the detection system as a part of a carbohydrate analyzer

The photometer measuring at 254 and 306 nm described in the previous section

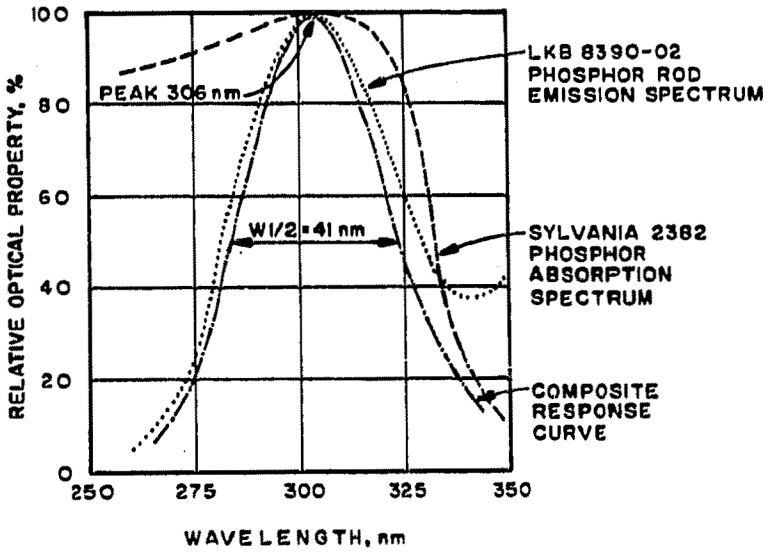

Fig. 3. The combination of phosphor-rod emission spectrum with phosphor absorption spectrum to provide effective detector response curve with 306-nm maximum.

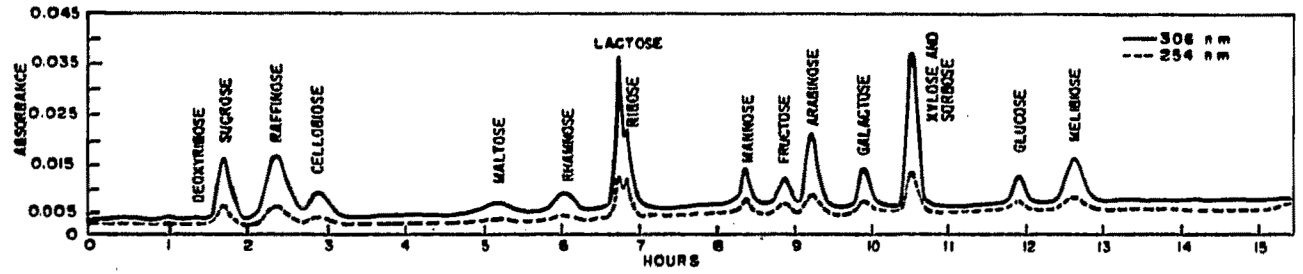

Fig. 4. Chromatogram of 1 ml of standard sugar solution containing 0.05 μmoles of each of 16 sugars.

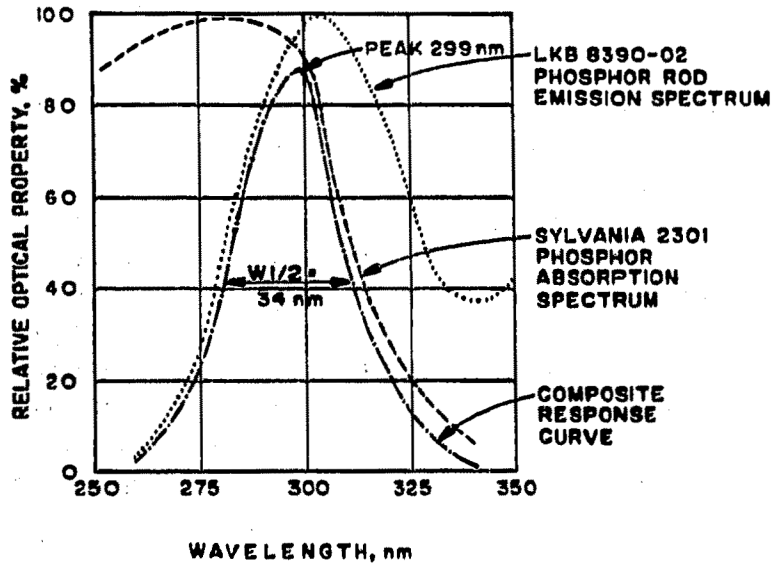

Fig. 5. The combination of phopshor-rod emission spectrum with phosphor absorption spectrum to provide effective detector response curve with 299-nm maximum.

was substituted for the colorimeter in the Mark III carbohydrate analyzer. A sulfuric acid flow of 19 ml/h and a reactor temperature of 100° were found to provide the maximum signal at 306 nm when using an 11 ml/h flow of an equimolar solution of sugars as substitute eluent. A series of sugars and body fluid samples were analyzed using these conditions.

The chromatogram for a 16-sugars standard containing 0.05 μmoles of each

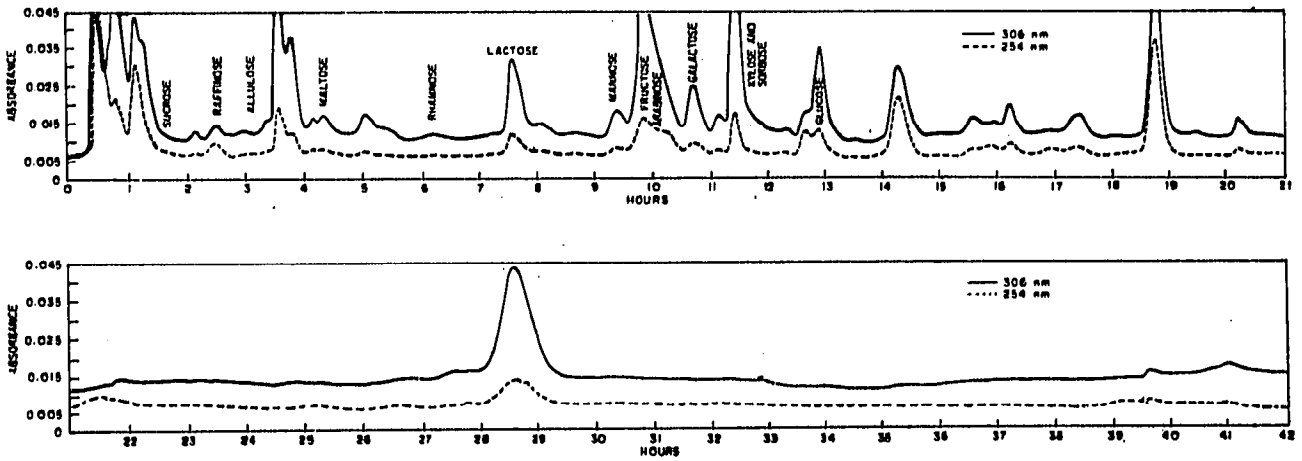

Fig. 6. Chromatogram of 1 ml of urine standard UR 2.

sugar was similar to that for the phenol-sulfuric acid reagent[23] (Fig. 4), except for small variations in relative peak sizes; however, because only two solutions are mixed, the new detection system provides a more stable base line. If interference were absent, a photometer using a 296-nm analytical channel would be more sensitive. An analytical channel with a peak absorption at 299 nm could be made for this purpose by using Sylvania phosphor type 2301 rather than type 2382 in the substituted filter and wavelength shifter (Fig. 5).

The chromatogram for a standard urine was similar to that obtained using the phenol–sulfuric acid reagent[23] (Fig. 6), except that the new system appears more sensitive to carbohydrate-related compounds eluting before and after the simple sugars.

The chromatogram for a dialyzed pooled blood serum (Fig. 7) shows better sensitivity than did a series of chromatograms prepared on an earlier model of the carbohydrate analyzer[26]. At least ten well-defined peaks are evident, with a number tentatively identified as shown.

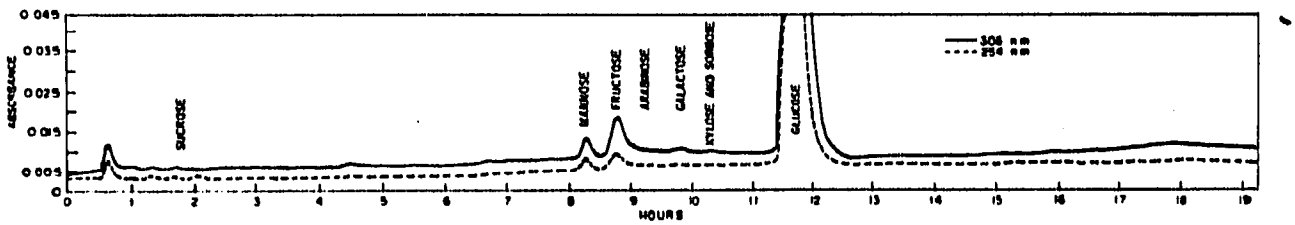

Fig. 7. Chromatogram of 1 ml of dialyzed pooled blood serum.

DISCUSSION AND CONCLUSION

The dynamic mixing of the column eluent with sulfuric acid followed by photometry is operationally simpler than any of the existing systems for detection of carbohydrates in eluents. Only one stable reagent is admixed to the eluent, and the reaction proceeds very rapidly. The limit of detection for sugars at least equals the 0.4–3.0 μg/ml reported for the phenol[3] reagent system. Discrimination from non-carbohydrates appears to be good because of the measurement at both 254 and 306 nm. However, as with other chromatographic separations of complex samples, the positive identification of the compounds responsible for the peaks often takes the application of additional techniques[27], and much additional work will be required to identify positively many compounds responsible for the peaks in the urine chromatograph of Fig. 6.

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