USE OF 4,5-DINITROVERATROLE FOR THE ULTRAMICRODETERMINATION OF REDUCING SUGARS ON PAPER CHROMATOGRAMS BY A REFLECTANCE METHOD

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Recently 4,5-dinitroveratrole was suggested as a sensitive qualitative test reagent for reducing sugars[1], and subsequently it was developed into a colorimetric method for the quantitative determination of reducing sugars in the range 0.5–3.0 mg[2]. Preliminary qualitative tests with this reagent showed that 1 μg of fructose, glucose, or arabinose could be detected on paper chromatograms. Further investigation showed that quantitative measurement of 2–20 μg of reducing sugars on paper chromatograms was possible. The experimental procedure and the results are presented.

MATERIALS AND METHODS

Standard solutions of arabinose, glucose, and fructose were prepared, separately, in concentrations of 2, 5, 10, 15, 20, and 25 μg per 5 μl of solution.

The 4,5-dinitroveratrole (4,5-d) was prepared according to the procedure of DRAKE *et al.*[3]. At time of use, as a dip reagent, a solution of 1 g 4,5-d per 100 ml of acetone was prepared.

One mole of potassium hydroxide was dissolved in sufficient 95% ethanol to make one liter of solution. This reagent should be freshly prepared and not stored for future use.

Using a micropipet, 5 μl aliquots of the sugar solutions were applied on Whatman No. 1, chromatographic grade, 18 × 22 inch paper. The chromatograms were irrigated by the descending procedure for 16 h at 25° with a solvent mixture of ethyl acetate–pyridine–water (8:2:1)[4]. After irrigation the chromatograms were removed from the cabinet and air-dried 30–60 min.

The chromatogram was first dipped into the 4,5-d reagent, then allowed to hang for 5–10 min, or longer if necessary, to permit complete volatilization of the acetone. The chromatogram was then dipped into the alcoholic KOH solution and again allowed to hang until the alcohol had completely evaporated from the sheet. The sheets were heated in an oven, in a humid atmosphere, at 60° for 10 min. Immediately after removing the chromatogram from the oven, the reflection densities of the sugar spots were measured with a Photovolt reflectance unit (Model 501-A)[5]* using light

* Mention of manufacturers and commercial products does not imply recommendation by the Department of Agriculture over others of a similar nature not mentioned.

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of 515 mμ wave length. Each spot was carefully scanned and the maximum density value recorded. The curve of each sugar for each chromatogram was obtained by plotting the amount of sugar (in the logarithmic direction) on semilogarithmic paper against the Photovolt reflection density readings.

RESULTS AND DISCUSSION

The reflection density readings of the sugar concentrations between 2 and 20 μg/5 μl of solution showed a linear relationship between the logarithm of the sugar concentration and the reflection reading (Fig. 1).

Because of the limitations of the uniformity of the spot densities, multiple spots of each sugar concentration should be applied to a given chromatogram[6,7]. To

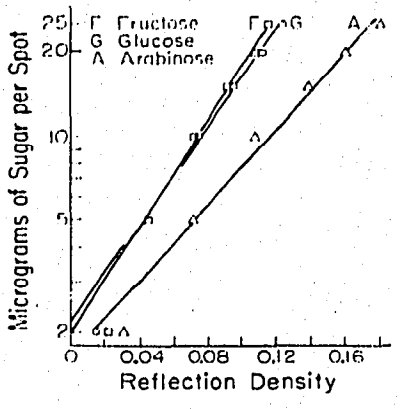

Fig. 1. Standard sugar curves, obtained by plotting amount of sugar, in the log direction on semilog paper, against photo volt reflection density readings using light of 515 mμ wave length.

further minimize differences due to variations from one sheet to another, a separate sugar curve should be prepared from each chromatogram, and the standard sugar curve and unknown sample should always be prepared on the same chromatogram. Adhering to these conditions each point on the curve between 2 and 20 μg can be reproduced within ± 1 μg.

The 4,5-d will give a qualitative test for reducing sugars at room temperature if the treated chromatogram is allowed to remain for several hours. However, to obtain the maximum color intensity, heat and a moist atmosphere are necessary. Variable time and temperature factors were studied and the optimum conditions were found to be 10 minutes at 60°.

The curves (Fig. 1) produced by the 6-carbon aldose and ketose-type sugars are similar whereas the aldopentose sugar curve has a different slope, primarily because this sugar produces with the 4,5-d reagent a more intensely colored spot of greater area than the aforementioned sugars.

Although numerous methods exist for the quantitative determination of sugars, only a few extend into the ultramicro range, and these do not distinguish an individual sugar in a sugar mixture. Therefore, the use of 4,5-d as a reagent for the determination

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of microgram quantities of individual sugars, via paper chromatograms, may find application.

SUMMARY

Using 4,5-dinitroveratrole as the chromogenic reagent, an ultramicro method has been developed for the quantitative measurement of 2–20 μg of reducing sugars on paper chromatograms.

ACKNOWLEDGEMENT

We are indebted to Mrs. N. FLOY BRACELIN for preparation of the figur

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Received September 13th, 1958