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QUANTITATIVE THIN-LAYER CHROMATOGRAPHY OF CARBOHYDRATES USING PENTAVALENT VANADIUM IN SULPHURIC ACID

DETERMINATION OF MIXTURES OF D-FRUCTOSE, D-GLUCOSE AND SUCROSE

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SUMMARY

Pentavalent vanadium in sulphuric acid can be used for the quantitative analysis of sugars. The yellow pentavalent vanadium is reduced by carbohydrates to the blue tetravalent form, and either the consumption of vanadium(V) or the amount of vanadium(IV) formed can be measured. For model studies, different D-fructose–D-glucose–sucrose mixtures were used. The monosaccharides were separated from the disaccharide by thin-layer chromatography. Owing to the great difference in reactivity between aldohexoses and ketohexoses, D-fructose and D-glucose can be determined separately without prior separation. The method enables 10 μg of each sugar to be determined.

INTRODUCTION

In many natural products, D-fructose, D-glucose and sucrose are the predominant mono- and disaccharides. The chromatographic separation of mono- and disaccharides is readily obtained[1], but D-fructose and D-glucose are not separated well in most chromatographic systems and the determination of each in a mixture is normally difficult and slow.

D-Fructose and D-glucose behave differently towards the oxidant vanadium(V) oxide in sulphuric acid, the former being oxidized rapidly by vanadium(V) whereas the latter is much more inert[1]. Malangeau *et al.*[2] have shown that vanadium(V) in 5 *M* sulphuric acid at 20° oxidizes ketoses and aldonic acids completely in a few hours, whereas no oxidation of aldoses was observed. Because of this difference in reactivity, it was suggested that the selective assay of aldonic acids or ketoses in the presence of aldoses in pharmaceutical preparations might be possible.

The yellow pentavalent vanadium is reduced by the sugars to the blue tetravalent state. The yellow form has an absorption maximum at 260 nm and the blue form at 700 nm. Either the consumption of vanadium(V) or the amount of vanadium(IV) formed can be measured. At 260 nm, the molar absorptivity of

vanadium(V) is 800 and of vanadium(IV) is 160; at 700 nm, the molar absorptivity of vanadium(IV) is 14, and vanadium(V) does not absorb at this wavelength. The measurement of the consumption of vanadium(V) is the most sensitive method, but measurement of the amount of vanadium(IV) formed is the simpler method.

EXPERIMENTAL

Materials

The oxidant (0.1 *M*) was prepared by dissolving ammonium monovanadate (11.7 g) in sulphuric acid (6 *M*, 100 ml). The spray reagent was 0.1 *M* vanadium(V) oxide in 1 *M* sulphuric acid[1] or 0.1 *M* ammonium monovanadate in 1 *M* sulphuric acid. The thin-layer plates were coated with silica gel G–Kieselguhr G (2:1) (0.25 mm), dried at 100° for 2 h, stored under ambient conditions in the laboratory and used without further activation. Toluene–acetic acid–methanol (2:2:1) was used as the solvent system.

Quantitative analysis

Macro method. A 50-μl volume of sample solution, containing 50–1000 μg of each sugar to be determined, is applied as a streak on a 20 × 20 cm thin-layer plate. The chromatogram is developed and dried and the sugars are located by spraying lightly with the spray reagent. The individual blue bands are scraped off into test-tubes, 5 ml of 0.1 *M* ammonium monovanadate solution in 6 *M* sulphuric acid are added to each and the tubes are shaken thoroughly. The suspension is filtered through a glass-fibre paper (Whatman GF/A). For the determination of D-fructose, the filtrate is left at room temperature for 30 min and the absorbance is read at 700 nm. For the determination of total monosaccharides, the solution is subsequently heated in a boiling water-bath for 20 min, before cooling and reading the absorbance at 700 nm. For sucrose and other "non-mixed" bands, the oxidation mixtures are also heated in a boiling water-bath for 20 min.

As a blank, a portion of equal area was scraped off from an adjacent sugar-free part of the chromatogram and treated as above.

Micro method. A 1-μl volume (Microcap pipettes; Drummond, Broomall, Pa., U.S.A.) of the sample solution, containing 5–50 μg of the sugars to be determined, is applied at three starting points on a 5 × 20 cm thin-layer plate. The chromatogram is developed and dried. To locate the sugars, two lanes are covered with a glass plate while the third lane is sprayed with the spray reagent. The required areas on the unsprayed lanes are located by comparison with the marker compounds in the detector lane. The required areas are scraped off into V-shaped tubes. To each tube are added 250 μl of 0.1 *M* ammonium monovanadate solution in 6 *M* sulphuric acid. To determine D-fructose alone, the tube containing a D-fructose–D-glucose mixture is left at room temperature for 30 min. For the determination of total monosaccharides and of sucrose, each tube containing these is heated in a boiling water-bath for 20 min. After the oxidation has been completed, the mixture is shaken thoroughly with 15 ml of water and filtered. The absorbance is measured at 260 nm against a reagent blank. The reagent blank is prepared by scraping off an equal area of an adjacent sugar-free part of the chromatogram and treating this as the test sample.

The absorbance with the micro-method is subtractive and not additive, as is

the "normal" case. The blank must be treated as the sample and the sample as the blank, *i.e.*, in a double-beam spectrophotometer the blank is placed in the sample beam and the sample in the reference beam. With a single-beam spectrophotometer, the absorbance is set at zero for the sample and the amount of oxidant consumed is found by reading the absorbance of the blank. Alternatively, the absorbance of the blank may be set at a given value, say 0.5, and the decrease in absorbance is measured.

Calculation. The amount of sugar is found from the appropriate calibration graph, where the chromatographic step has been included. For mixtures of D-fructose and D-glucose, the values to be used in the calibration graph must be calculated from the absorbance values (macro method) or the decrease in absorbance values (micro method) found by oxidizing at 20° and 100° (see below).

RESULTS AND DISCUSSION

The rate of oxidation of carbohydrates with vanadium(V) is strongly dependent on the sulphuric acid concentration, the rate increasing rapidly with increasing acid concentration[1,3]. To obtain a complete oxidation in a short time, high acid concentrations are chosen. To find conditions where the D-fructose and D-glucose contents of mixtures could be determined separately, the sulphuric acid concentration, the reaction period and the temperature were varied. Ideally, the conditions should be such that (a) fructose is completely oxidized and glucose not oxidized at all, and (b) glucose is also completely oxidized. To be of practical routine use, the method should not be time consuming. Fig. 1 shows the absorbances obtained when oxidizing D-fructose and D-glucose at 20° with ammonium monovanadate at different sulphuric acid concentrations *versus* time, and Fig. 2 shows the absorbances obtained on oxidizing D-fructose and D-glucose at 100° for 20 min and 20° for 30 min *versus*.the sulphuric acid concentration.

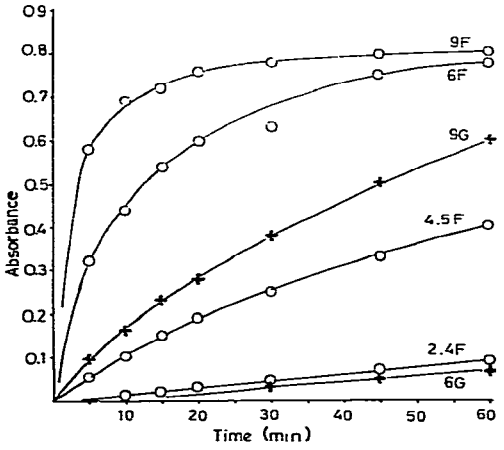

Fig. 1. Absorbance measured at 700 nm *versus* time for the oxidation of 900 μg of D-fructose and D-glucose in 2.5 ml of 0.1 *M* ammonium monovanadate solutions of different sulphuric acid concentrations. Temperature, 20°. 6G = D-glucose (+) in 6 *M* sulphuric acid; 2.4F = D-fructose (○) in 2.4 *M* sulphuric acid; etc.

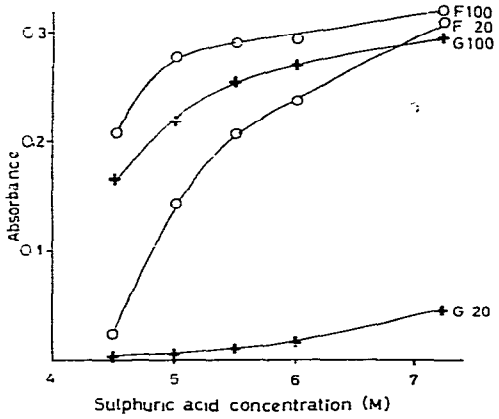

Fig. 2. Absorbance measured at 700 nm *versus* sulphuric acid concentration for the oxidation of 360 μg of D-fructose and D-glucose in 2.5 ml of 0.1 *M* ammonium monovanadate at different sulphuric acid concentrations, treated for 30 min at 20° and for 20 min at 100°. F 100 = D-fructose (○) oxidized at 100°; G 20 = D-glucose (+) oxidized at 20°; etc.

The ideal conditions were not found. However, the conditions where $A_F^{100} - A_G^{20}$ is at a minimum while $A_F^{100} - A_G^{20}$ is at a maximum gives the closest approach to the ideal conditions (where A_F^{100} is the absorbance obtained on oxidizing D-fructose at 100° and A_G^{20} is the absorbance obtained on oxidizing D-glucose at 20°). This was obtained for a sulphuric acid concentration of 6 *M*, a reaction period of 30 min and a temperature of 20° [condition (a)], and 6 *M* sulphuric acid for 20 min at 100° [condition (b)].

The absorbance on oxidizing D-fructose under condition (a) was found to be 85% of that found under condition (b):

$$A_F^{20} = 0.85\, A_F^{100} \tag{1}$$

and the absorbance on oxidizing D-glucose under condition (a) was 5% of that obtained under condition (b):

$$A_G^{20} = 0.05\, A_G^{100} \tag{2}$$

With the oxidant in excess, it was found that at both temperatures the total absorbance (A_{F+G}) for a mixture of D-fructose and D-glucose was additive:

$$A_{F+G}^{20} = A_F^{20} + A_G^{20} \tag{3}$$

$$A_{F+G}^{100} = A_F^{100} + A_G^{100} \tag{4}$$

Solving these four simple equations with four unknowns gives the following solutions:

$$A_F^{100} = \frac{A_{F+G}^{20} - 0.05\, A_{F+G}^{100}}{0.80} \tag{5}$$

$$A_G^{100} = \frac{0.85\,A_{F+G}^{100} - A_{F+G}^{20}}{0.80} \quad (6)$$

After calculating the absorbance contributed by each component, the amount of D-fructose and D-glucose originally present in the mixture is found from the respective calibration graphs, which are obtained by oxidizing each compound at 100°. The amount of sucrose is found from its calibration graph using the observed absorbance, A_S^{100}.

Tables I and II shows the results obtained on analyzing different mixtures of D-fructose, D-glucose and sucrose according to the macro and the micro methods. The macro method is the simplest and is to be preferred if a sufficient amount of sugar is available (>50 μg). The method is convenient as the oxidant is used as the spray reagent. After localization, scraping off the blue band and addition of more oxidant, the oxidation is continued to completion. The method ensures that all of the substance to be measured can be scraped off, as the band is visible, but it also avoids the inclusion of other compounds, which is advantageous if the sugar has moved unevenly on the chromatogram, giving a distorted band. In the analysis of D-fructose–D-glucose mixtures, the total amount of hexoses at 100° is determined on the same

TABLE I

QUANTITATIVE ANALYSIS OF MIXTURES OF D-FRUCTOSE, D-GLUCOSE AND SUCROSE BY THE MACRO METHOD

The following amounts were streaked on the thin-layer plate (micrograms of each sugar in the order D-fructose–D-glucose–sucrose): A, 100:900:100; B, 500:500:500; C, 900:100:900. Six analyses were carried out on each mixture. The mean recovery, the standard deviation (*s*) and the standard deviation as a percentage are given.

Parameter	D-*Fructose*			D-*Glucose*			*Sucrose*		
	A	*B*	*C*	*C*	*B*	*A*	*A*	*B*	*C*
Amount added (μg)	100	500	900	100	500	900	100	500	900
Mean recovery (μg)	96	490	885	110	480	880	120	530	900
s (μg)	20	35	40	55	45	40	25	40	40
s (%)	20	7	5	50	10	5	20	7	5

TABLE II

QUANTITATIVE ANALYSIS OF MIXTURES OF D-FRUCTOSE, D-GLUCOSE AND SUCROSE BY THE MICRO METHOD

The following amounts were spotted on the thin-layer plate (micrograms of each sugar in the order D-fructose–D-glucose–sucrose): A, 10:40:10; B, 25:25:25; C, 40:10:40. Six analyses were carried out on each mixture. The mean recovery, the standard deviation (*s*) and the standard deviation as a percentage are given.

Parameter	D-*Fructose*			D-*Glucose*			*Sucrose*		
	A	*B*	*C*	*C*	*B*	*A*	*A*	*B*	*C*
Amount added (μg)	10	25	40	10	25	40	10	25	40
Mean recovery (μg)	12.5	26.0	38.2	19.5	26.2	42.0	10.0	25.3	29.5
s (μg)	2.5	2.5	5	10	2.5	4	3	4	3
s (%)	20	10	13	50	10	10	33	16	7

sample as was used for the determination at 20°. When D-glucose is the only hexose present, or is present in a large excess, some of it will be oxidized on the plate after spraying. The amount of blue colour developed will be measured as D-fructose. However, the "detection time" will reveal if D-fructose is present[1]. Also, on spraying lightly with the spray reagent only a small portion of D-glucose present is oxidized, thus minimizing the error. On the 20 × 20 cm thin-layer plates, a maximum load of 1200 μg of D-fructose plus D-glucose could be separated from 1200 μg of sucrose.

The micro method can be used with spots of 1-μl volumes on the thin-layer plates, allowing several analyses to be carried out per plate. The procedure is less convenient than the macro method, and the standard deviation is larger. The determination of D-fructose and total hexoses must be performed on two separate test mixtures. A maximum load of 60 μg of D-fructose plus D-glucose could be separated from 60 μg of sucrose in the system used, with a single spotting.

A linear response was found for both methods, in the range 0–1200 μg of sugar for the macro method and 0–60 μg of sugar for the micro method (excess of oxidant).

In mixtures of D-fructose and D-glucose where D-fructose is present in large excess (mixture C in Tables I and II), the determination of D-glucose is unreliable, as would be expected by inspection of eqn. 6. Both A_{F+G} and A_{F+G} have large values, the difference between them being small with a relatively large uncertainty.

The accuracy of quantitative analysis on thin-layer chromatograms is not generally considered to be high[4]. Performing the oxidations on pure samples and omitting the thin-layer chromatographic step gave a much lower standard deviation of the results. Undoubtedly, a much lower standard deviation could be obtained if the procedure for preparation and handling of plates is rigorously standardized[4].

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