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Study of carbohydrates with cysteine-sulfuric acid reagents.

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STUDY OF CARBOHYDRATES WITH CYSTEINE-SULFURIC ACID REAGENTS

BY

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ABSTRACT

A modified procedure for the use of the cysteine-sulfuric acid reagent for the determination of monosaccharides has been devised. The procedure utilizes a 9-ml volume of H_2O:H_2SO_4 at a ratio of 1:7 H_2O:H_2SO_4 which results in a rapid, non-specific method for the determination of pentoses, hexoses, and hexuronic acids in simple solutions. Alpha-substituted cysteines are compared to cysteine hydrochloride as colour reagents. The accuracy of the modified procedure is about $\pm$ 2% in the optimum concentration range. The time required for a complete analysis is about one hour for pentoses and hexuronic acids and thirty minutes for hexoses.

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CHAPTER I

INTRODUCTION

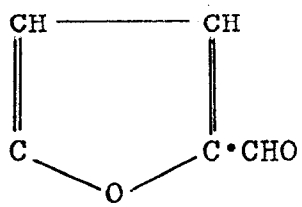
Several colorimetric methods for the qualitative and quantitative determination of reducing sugars have been reported (1). Group specific colour reactions for carbohydrates are based on their ability to form furfural or its homologues in strong acids, particularly after heating (2,3). These furan derivatives or their reaction products derived from oxidation, reduction, or condensation processes in strong acids can form coloured products either with the sugars themselves, or with organic substances. The reagents such as 1-naphthol (4) for carbohydrates in general, benzidine for pentoses and uronic acids (5-7), naphthoresorcinol for uronic acids (8,9), resorcinol (10), carbazole (11), and sulfhydryl compounds (1,12) are well known examples of colorimetric tests that may be carried out in acid media. Such tests as these have recently gained added importance since the extensive development of partition chromatography for the separation and characterization of minute amounts of sugars and their derivatives (13-18).

Volumetric procedures involving the use of potassium ferricyanide (19), ceric sulfate (20), copper sulfate (21), and sodium hypoiodite (22), are applicable to the determination of small amounts of reducing sugars after

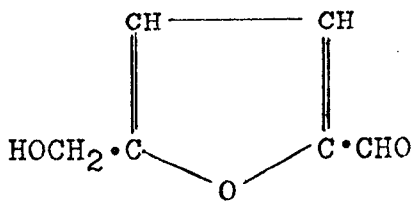
separation by partition chromatography. However, these methods require considerable skill, are time-consuming and sensitive to slight variation in the conditions (23).

One of the classical colorimetric procedures which has been frequently studied and modified is that employing a cysteine-sulfuric acid reagent for the determination of sugars (12). This reagent has been applied to the qualitative and quantitative determination of hexoses, methyl pentoses, pentoses, hexuronic acids, and tetroses (24-30).

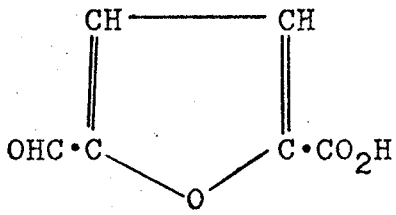
The method consists of treating the carbohydrate-containing material with sulfuric acid. The ultraviolet absorption spectra of strong H_2SO_4 solutions of pentoses and hexoses have been identified, respectively, with the spectra of furfural and 5-hydroxymethyl-2-furfuraldehyde (31-33). The expected products from uronic acids are furfural, reductic acid (2,3-dihydroxy-2-cyclopenten-1-one), and 5-formyl-2-furoic acid (34,35). It is important to note that there are other reaction products in addition to the major products mentioned. The structures of the major reaction products are illustrated below:



furfuraldehyde

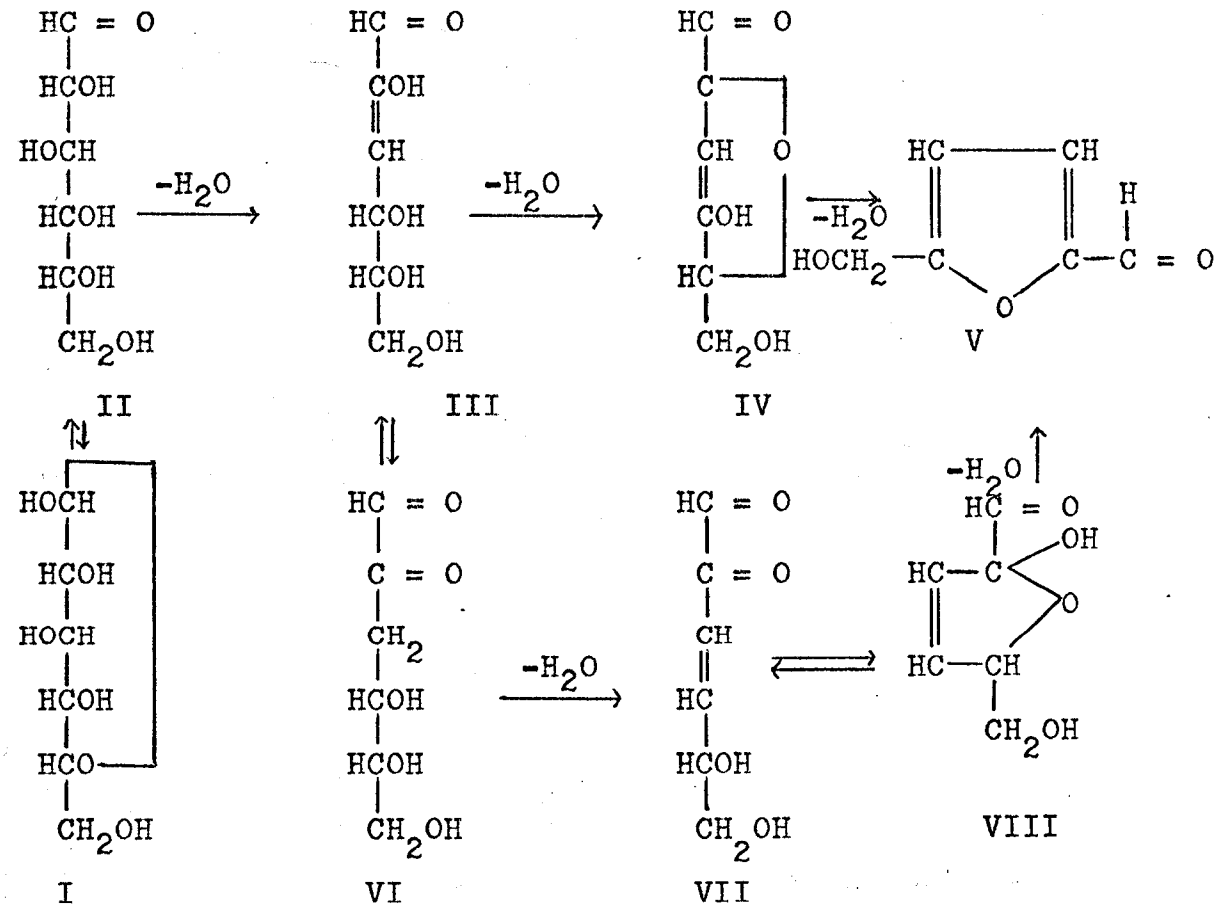


5-hydroxymethyl-2-furfuraldehyde



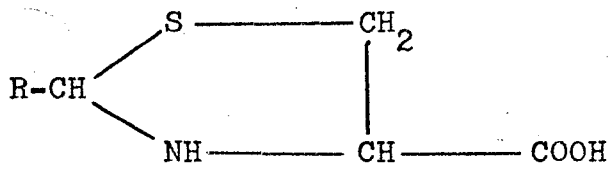
5-formylfuroic acid

The mechanisms of the reactions involving the sugars with sulfuric acid have not all been established. Wolfrom *et al.* (2) has postulated a mechanism for the conversion of glucose to 5-hydroxymethyl-2-furfuraldehyde which involves dehydration of an hydroxyl in the β position to a carbonyl. The proposed reactions are as follows:



D-glucose, represented by I, is transofmred first into II or its aldehydrol. The intermediate III then results from II by loss of water, producing a conjugated enol. The postulated intermediate III could produce IV by cyclic dehydration and finally V could result from IV by final dehydration producing a third double bond in conjugation with the two already present.

The final reaction products form condensation products with cysteine hydrochloride which is the colour reagent employed by Dische. Cysteine combines with aldehydes in a mole to mole ratio with elimination of a mole of water (36,37). The structure proposed for these compounds is as follows (36):



The symbol (R) represents the reaction products formed as a result of reacting the carbohydrates with sulfuric acid.

Dische utilizes the cysteine-sulfuric acid reagent for the determination of monosaccharides in biological materials. The procedure usually requires considerable time of standing due to the instability of the reaction products, in both the qualitative and quantitative determination of carbohydrates (1). The stability of

the reaction products is attributed to the formation of intermediates other than the major substances already mentioned (24,38). Dische and several other investigators have observed that the concentration of sulfuric acid influences three aspects of the analysis: the position and intensity of the maximum; the course of the reaction; and stabilities of the reaction products (26, 38-41). Bandow (31,42) reported a shift of the ultraviolet absorption spectrum of furfural to longer wavelengths and an increase in molar absorption with increasing sulfuric acid concentration. A similar shift was observed by Love (33) in the reaction of glucose with sulfuric acid. Love (33) in an attempt to explain this shift and the increase in sensitivity suggested that the reaction products other than furfural and its derivatives are altered in concentrated sulfuric acid in such a way as not to interfere with the colour development involving furfural and its derivatives. Experimental evidence to support this was provided by Bandow (31) who showed that the furfural molecule was not radically altered in concentrated sulfuric acid.

The object of this study is to investigate the effect of varying the concentration of sulfuric acid and the volume of the acid mixture on the cysteine hydrochloride reagent for the determination of carbohydrates in simple solutions with a view to obtaining greater stability, speed,

absorbancy, and accuracy. The effect of α-substituted cysteines which were synthesized in this laboratory (43) will be compared to cysteine hydrochloride as colour reagents.

CHAPTER II

EXPERIMENTAL

A. Modified Procedure with Cysteine Hydrochloride

Materials and Methods

1) Reagents

Cysteine hydrochloride was obtained from Fisher Scientific Company. The sugars employed were all analytical grade, also obtained from Fisher Scientific Company. All sugar solutions were made up in deionized water. Reagent grade sulfuric acid (A.C.S. specification) was used.

2) Apparatus

Absorbance measurements were made with a Beckman DU monochromator equipped with a Gilford Model 220 absorbance indicator, a Gilford Model 210 automatic cuvette positioner, a Sargent Model SR Recorder, and matched 1-cm quartz cells. Heating and cooling was controlled by constant temperature water baths set at 25 ± 0.1° and 100° ± 1°C. The absorbance measurements reported are the average values of triplicate analyses.

3) Effect of Sulfuric Acid Concentration and Volume of Acid

The amount of cysteine hydrochloride was maintained at 0.1 ml of a 3% solution (w/v) and the quantity of hexoses

at 100 µg of glucose per ml. Different ratios of water to sulfuric acid were studied and the volume of these acid mixtures varied from 4.5 to 12 ml. The procedure followed was basically that of Dische's primary cysteine reaction (24): The acid mixtures were added to 25 x 150 mm test tubes immersed in an ice bath. After allowing to cool, 1 ml of sugar solution was added slowly and the mixtures were shaken to ensure complete mixing. They were heated for exactly 3 min in a boiling water bath after they had been brought to room temperature in a water bath at 25°C. After allowing to cool to room temperature, 0.1 ml of a 3% solution (w/v) of cysteine hydrochloride was added and the mixtures were shaken. Spectral and stability studies were performed on the sugar mixtures.

4) Effect of Time of Heating

The procedure described above was carried out on pentose, hexose, and hexuronic acid solutions. The solutions were heated for different times in the boiling water bath in order to obtain the optimum heating times for maximum colour development.

5) Stability of Reaction Products

The stability of the reaction products for the various sugars was studied by recording the change of absorbance with time at the appropriate maxima for each sugar and under the optimum reaction conditions with respect to time of heating. Pentoses, hexoses, and

hexuronic acids were kept in the boiling water bath for 15, 3, and 15 min, respectively.

6) Modified Procedure for Hexoses

A mixture (9 ml) of 1 part H_2O and 7 parts H_2SO_4 (made up by adding 210 ml of concentrated H_2SO_4 to 30 ml of H_2O) was added to 25 x 150 mm test tubes immersed in an ice bath. After allowing a few minutes to cool, 1 ml of sugar solution was added slowly into the test tubes. The test tubes were then shaken to ensure complete mixing. The test tubes were then placed in a water bath at 25°C for a few minutes (3-5 min) and then for exactly three minutes in a boiling water bath at 99-100°C. The tubes are cooled to room temperature in the water bath at 25°C. After this cooling period (approximately 3 min) 0.1 ml of a 3% solution (w/v) of cysteine hydrochloride was then added and the mixtures were shaken. The mixtures were allowed to stand at room temperature for 15 min, after which the absorbance of the solutions were read at 410 mμ. (The solutions were periodically shaken during the time of standing in order to avoid formation of bubbles.)

7) Modified Procedure for Pentoses and Hexuronic Acids

The procedure for the determination of pentoses and hexuronic acids is different with regards to absorption maxima, time of heating, and time of standing after the addition of colour reagent. For pentoses absorbance measurements were read at 390 mμ after allowing the

solutions to stand for 35 min after the addition of cysteine hydrochloride. For glucuronic acid and galacturonic acid absorbance measurements were read at 390 and 395 mμ, respectively, after allowing the solutions to stand for 30 min after the addition of the colour reagent.

B. Alpha-Substituted Cysteines as Colour Reagents

Materials and Methods

1) Reagents and Apparatus

All reagents and apparatus employed were mentioned earlier except for the α-substituted cystines which were synthesized in this laboratory (43). Na-Hg amalgam (1%) was utilized for the reduction of the cystines to cysteines (44).

2) Reduction of Cystines with Na-Hg Amalgam

The Na-Hg amalgam (1%) was prepared by the method of Holleman (43) and stored under toluene. Deionized water (5 ml) was added to the washed amalgam (10 gm in a 50-ml beaker). Sufficient solid α-substituted cystines to prepare a 3% solution (w/v) were added to the beaker and covered with a watchglass to prevent loss due to spattering. The cystines are not soluble in water but as the reduction progresses they all dissolve due to the conversion of cysteine which is soluble in water. The solutions were quantitatively transferred to a 25-ml volumetric flask with deionized water.

3) Effect of Alpha-Substituted Cysteines

All experimental conditions were kept the same as in the modified procedure except for the colour reagents. [The α-substituted cysteines were 3% solutions (w/v).] The effect of the α-substituted cysteines on the absorption maxima, time of standing, and absorbance was studied.

CHAPTER III

RESULTS

A. Modified Procedure with Cysteine Hydrochloride

1) Effect of Sulfuric Acid Concentration and Volume of Acid

The results of the variation of sulfuric acid concentration and volume of acid mixtures are shown in Table I. These variations were studied with respect to wavelength (λ), absorbance (A), and stability (S) of the reaction products after the addition of colour reagent. The absorbance readings were corrected using a blank of 1 ml of water instead of sugar solution.

2) Effect of Time of Heating

The concentration of the primary and final reaction products depends on the time during which the reaction mixture remains at the temperature necessary for the decomposition of the sugar. Pentoses, hexoses, and hexuronic acids were heated for different times in a boiling water bath in order to establish the optimum times of heating. The hexoses, particularly glucose and fructose, became a light pink colour after heating in a boiling water bath (99-100^{o}C) for any time intervals over 3 min. The optimum times of heating for the various classifications of monosaccharides are listed in Table II.

TABLE I

EFFECT OF SULFURIC ACID CONCENTRATION AND VOLUME OF ACID MIXTURE

$H_2O:H_2SO_4$ ratio	Volume 4.5			6			9			12		
	λ	A	S	λ	A	S	λ	A	S	λ	A	S
1:5	410	0.657	dec.*	405	0.811	dec.	415	0.817	slowly dec.	400	0.714	stable
1:6	410	0.918	dec.	410	1.072	dec.	408	0.965	slowly dec.	410	0.848	stable
1:7	410	1.066	dec.	410	1.194	dec.	410	1.066	stable	410	0.880	stable

* decreasing

TABLE II

OPTIMUM TIMES OF HEATING

Classification	Sugar	Absorption maxima (mμ)	Time of heating (min)
Pentoses	Xylose Ribose	390	15
Hexoses	Glucose Fructose Galactose Mannose	410	3
Hexuronic acids	Glucuronic Galacturonic	390 395	15

3) Spectral Studies of Sugars Employing the Modified Procedure

The modified procedure was applied to all sugars. The absorption spectra for the different classifications of sugars was obtained by taking absorbance readings at 10 mμ intervals and at 5 mμ intervals when approaching the maximum. The results are illustrated in Figures 1-3 and the absorption maxima are listed in Table II.

4) Stability Studies

The stability of the reaction products was followed by recording the change of absorbance with time. The results of this study are illustrated in Figure 4.

5) Modified Procedure for Carbohydrates

The modified procedure was applied to the determination of hexoses, pentoses, and hexuronic acids. The differences in the procedures for the determination of various classifications of sugars is listed in Table III. A linear relationship of absorbance to the concentration of sugar was observed for all sugars investigated using the modified procedures. The results of this study are shown in Table IV. The values shown are the average values of triplicate analyses. Beer's Law plots for three of the sugars studied are illustrated in Figure 5. The accuracy and reproducibility of the various Beer's Law plots were determined (Table V).

FIGURE I

ABSORPTION SPECTRA OF PENTOSES

Legend

Spectra of various pentoses treated with the modified procedure. The spectra were obtained 15 min after the addition of cysteine hydrochloride: Curve 1, 80 μg of xylose; Curve 2, 50 μg of ribose.

FIGURE 1

ABSORPTION SPECTRA OF PENTOSES

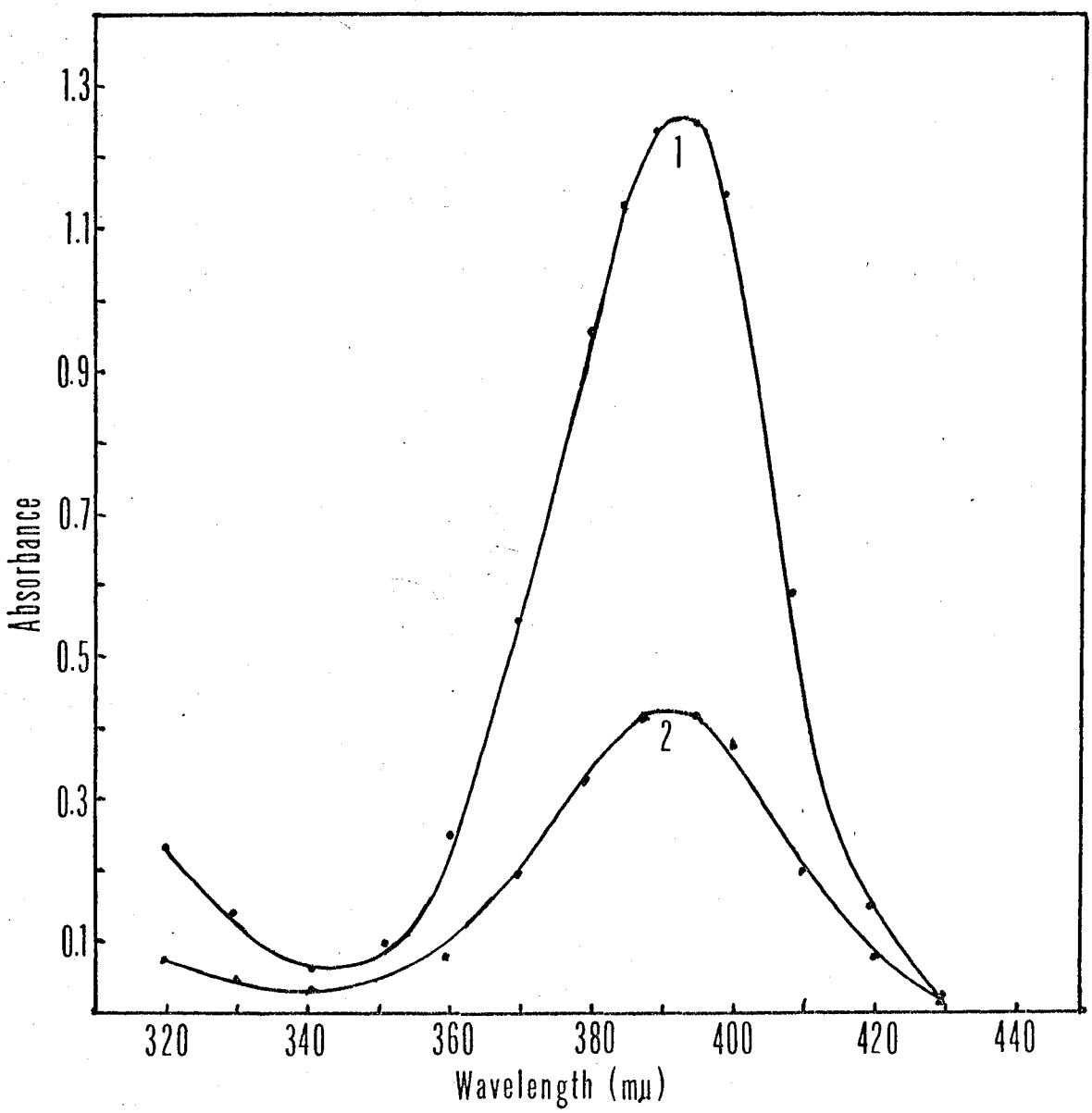


FIGURE 2

ABSORPTION SPECTRA OF HEXOSES

Legend

Spectra of various hexoses obtained after 3 min heating at 99-100°C and ten min after the addition of Cysteine hydrochloride. Curve 1, 50 µg glucose; Curve 2, 50 µg fructose; Curve 3, 50 µg mannose; Curve 4, 50 µg galactose.

FIGURE 2

ABSORPTION SPECTRA OF HEXOSES

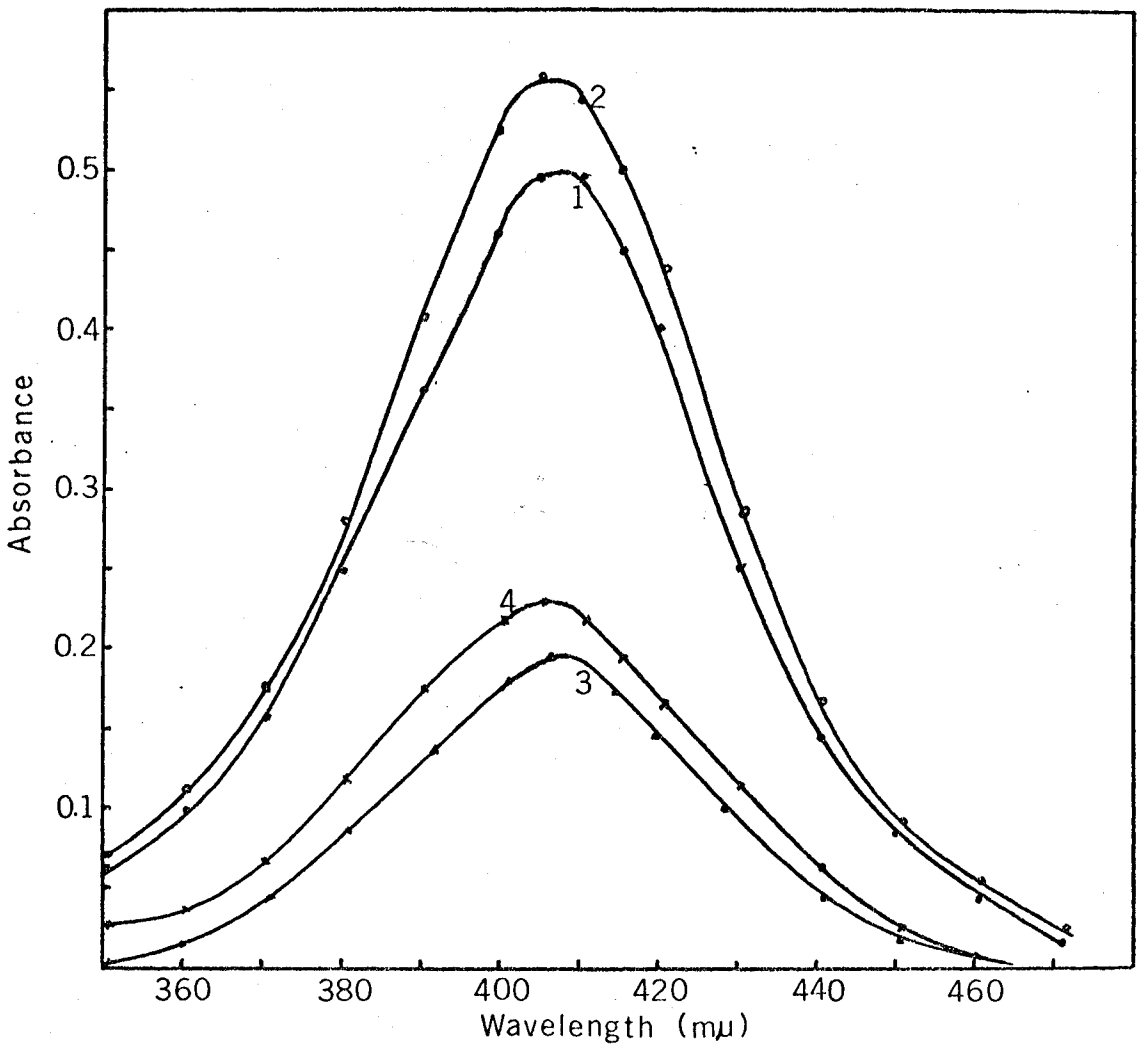


FIGURE 3

ABSORPTION SPECTRA OF HEXURONIC ACIDS

Legend

Spectra of various hexuronic acids obtained after 15 min heating at 99-100°C and 15 min after the addition of cysteine hydrochloride. Curve 1, 100 µg glucuronic acid; Curve 2, 200 µg galacturonic acid.

FIGURE 3

ABSORPTION SPECTRA OF HEXURONIC ACIDS

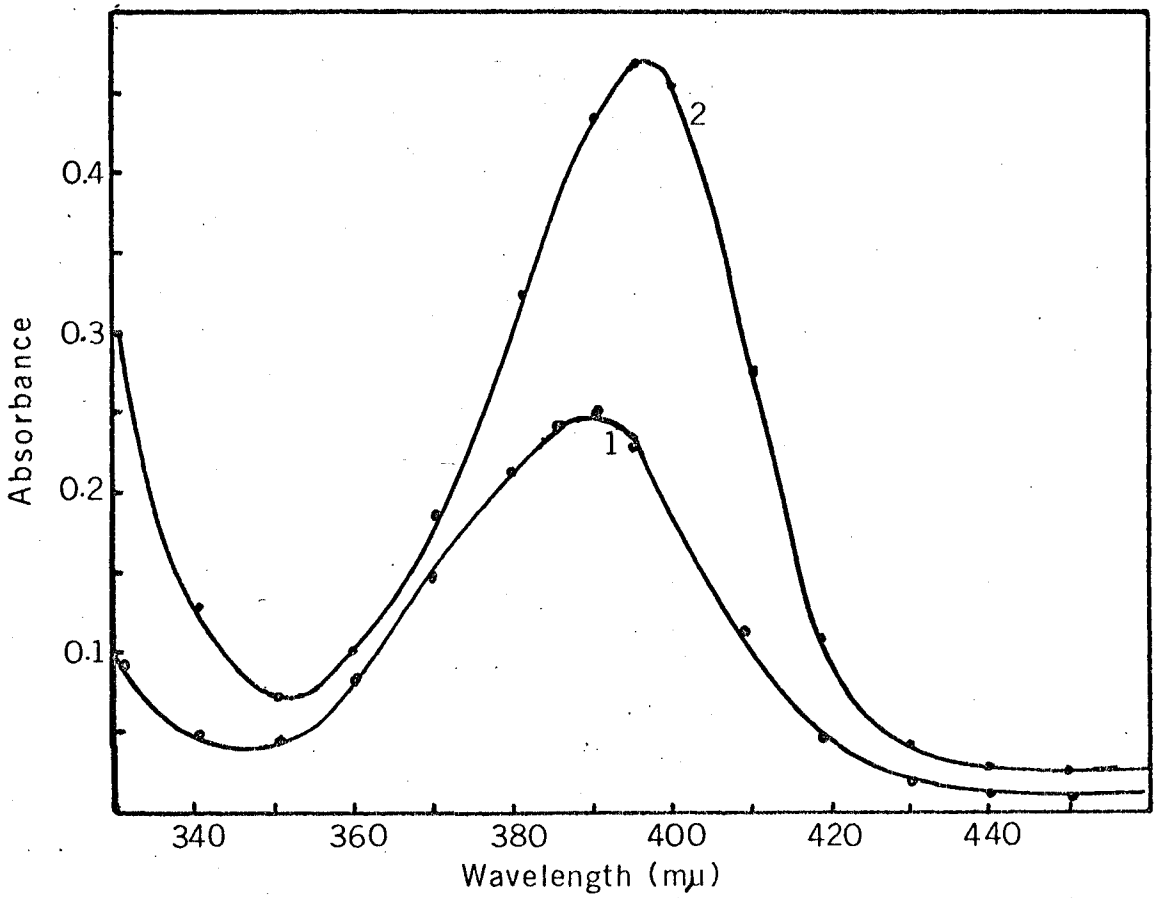


FIGURE 4

STABILITY STUDY

Legend

Change of absorbance with time: Curve 1, 80 µg xylose, 390 mµ; Curve 2, 50 µg ribose, 390 mµ; Curve 3, 50 µg mannose, 410 mµ; Curve 4, 50 µg galactose, 410 mµ; Curve 5, 50 µg glucose, 410 mµ; Curve 6, 50 µg fructose, 410 mµ; Curve 7, 100 µg glucuronic acid, 390 mµ; Curve 8, 200 µg galacturonic acid, 395 mµ.

FIGURE 4

STABILITY STUDY

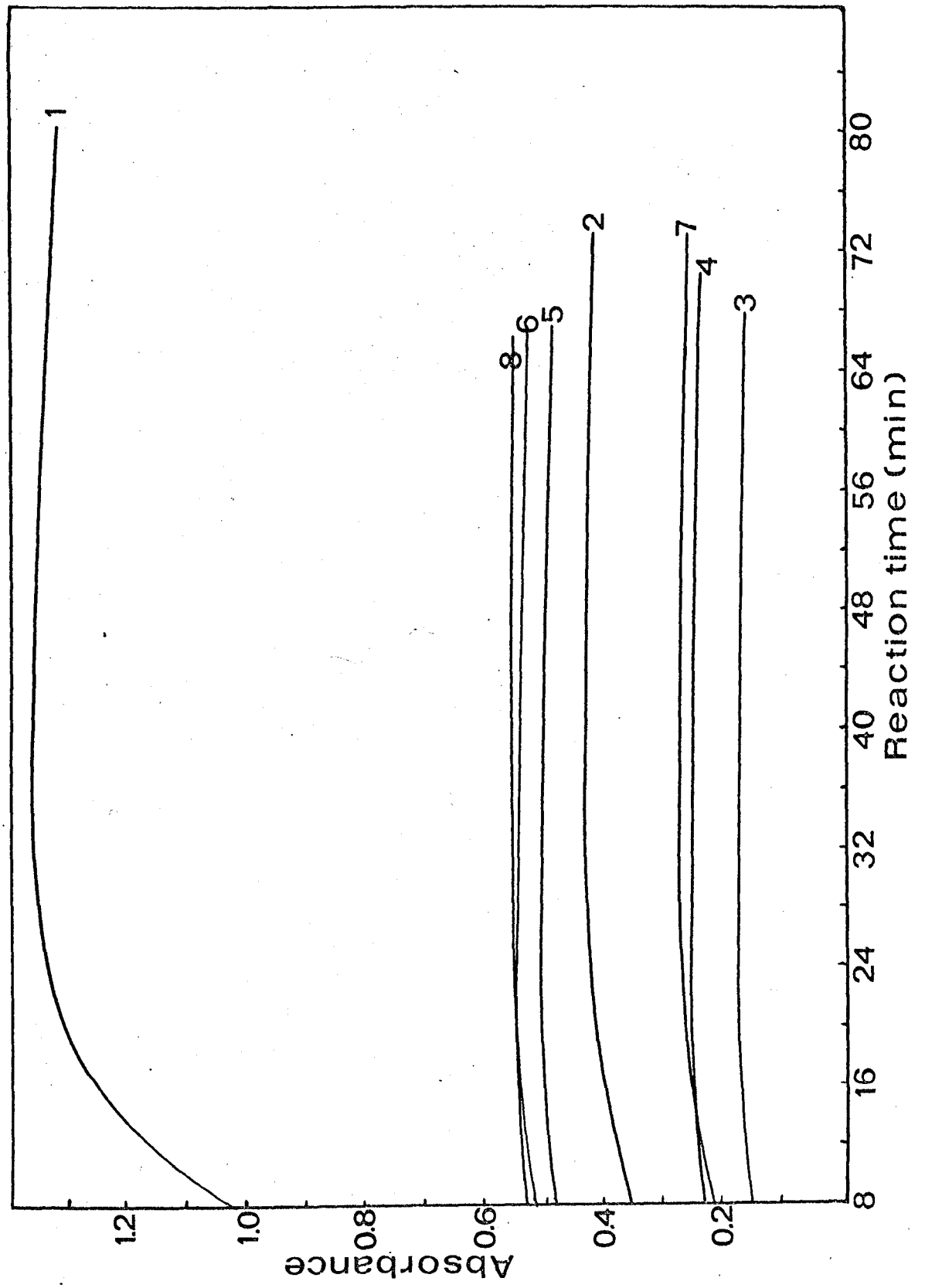


TABLE III

PROCEDURE FOR DETERMINATION OF SUGARS

Classification	Sugar	Absorption maxima (mμ)	Time of heating (min)	Time of standing (min)
Pentoses	Xylose	390	15	35
	Ribose			
Hexoses	Glucose	410	3	15
	Fructose			
	Galactose			
	Mannose			
Hexuronic acids	Galacturonic	395	15	30
	Glucuronic	390		

TABLE IV

BEER'S LAW STUDY OF SUGARS

Sugar	Concentration (µg/ml)	Absorbance	*Average deviation (±)
Xylose	8	.146	.004
	16	.303	.005
	32	.618	.004
	40	.754	.006
	56	1.048	.010
	72	1.314	.002
	80	1.443	.003
Ribose	15	.143	.002
	30	.291	.005
	45	.439	.005
	60	.586	.006
	70	.670	.003
	80	.766	.006
	90	.846	.006
	100	.939	.002

.. continued

* in absorbance units

Table IV(continued)

Glucose	15	.163	.005
	30	.312	.003
	45	.482	.004
	60	.645	.004
	70	.747	.004
	80	.849	.006
	90	.945	.003
	100	1.066	.002
Fructose	10	.128	.005
	20	.242	.003
	30	.359	.004
	40	.466	.003
	50	.574	.000
	60	.710	.004
	80	.936	.002
	100	1.170	.004
Mannose	20	.087	.005
	40	.166	.003
	60	.249	.005
	80	.345	.004
	100	.427	.003
	120	.515	.002

.. continued

Table IV (continued)

	150	.640	.003
	160	.675	.005
Galactose	16	.089	.002
	30	.156	.004
	40	.202	.002
	60	.309	.004
	80	.414	.003
	100	.515	.003
	140	.725	.004
	180	.932	.005
Glucuronic acid	20	.053	.004
	40	.114	.003
	60	.172	.002
	80	.236	.005
	100	.295	.004
	120	.348	.004
	150	.449	.001
	180	.525	.004
	200	.607	.005
Galacturonic acid	20	.062	.010
	40	.119	.006
	60	.170	.005

.. continued

Table IV (continued)

80	.231	.005
100	.275	.010
120	.332	.005
140	.390	.006
180	.507	.004
200	.567	.005
220	.620	.003
250	.706	.002
280	.782	.008

FIGURE 5

TYPICAL BEER'S LAW PLOTS

Legend

Linear response of absorbance to the amount of sugar in 1-ml sample: Curve 1, xylose, 390 mμ; Curve 2, glucose, 410 mμ; Curve 3, glucuronic acid, 390 mμ.

FIGURE 5

TYPICAL BEER'S LAW PLOTS

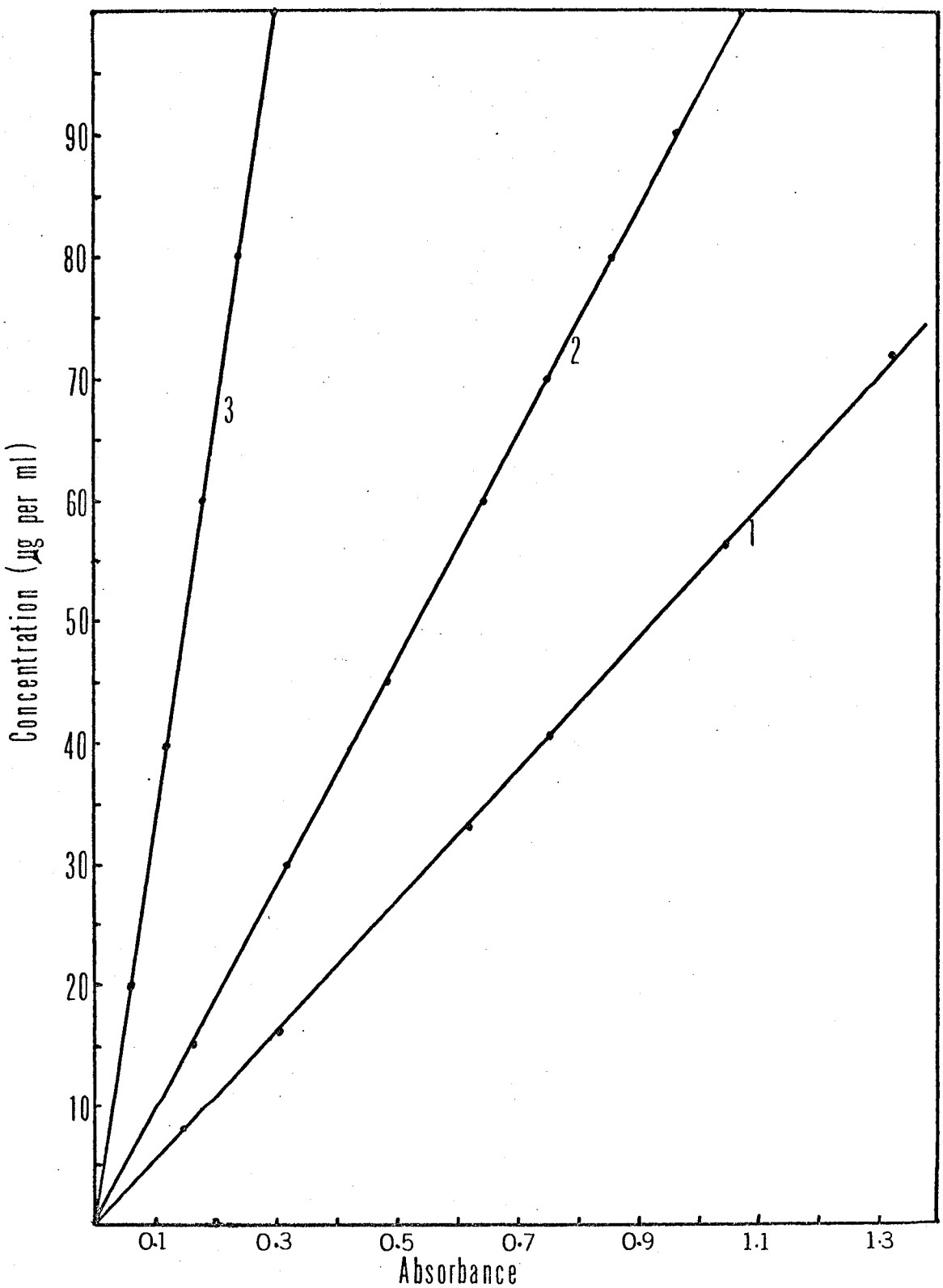


TABLE V

ACCURACY AND REPRODUCIBILITY OF BEER'S LAW

PLOTS EMPLOYING THE MODIFIED PROCEDURE

Legend

Average deviation calculated in absorbance units.

TABLE V

ACCURACY AND REPRODUCIBILITY OF BEER'S LAW PLOTS EMPLOYING THE MODIFIED PROCEDURES

Sugar	Amount added (μg)	Absorbance (average)	Deviation (±)	Amount recovered (μg)	% error
Xylose	24	0.458	0.002	24.5	+2.08
	48	0.895	0.008	48.0	0.00
	64	1.170	0.004	62.8	-1.88
Ribose	25	0.236	0.002	24.5	-2.00
	50	0.485	0.002	50.5	+1.00
	75	0.723	0.006	75.5	+0.67
Glucose	20	0.211	0.001	20.0	0.00
	40	0.439	0.004	41.0	+2.50
	50	0.556	0.004	52.0	+4.00
Fructose	20	0.237	0.006	20.2	+1.00
	50	0.589	0.003	50.5	+1.00
	90	1.056	0.002	90.5	+0.55
Mannose	40	0.165	0.005	38.9	-0.25
	70	0.303	0.004	70.5	+0.72
	90	0.378	0.002	89.0	-1.11
Galactose	40	0.193	0.000	38.0	-5.00
	70	0.351	0.004	68.5	-2.14
	90	0.463	0.004	90.0	0.00
Glucuronic acid	80	0.237	0.004	80.0	0.00
	150	0.451	0.001	152	+1.33
	180	0.591	0.005	183	+1.67
Galacturonic acid	50	0.141	0.003	50.0	0.00
	120	0.345	0.001	121.5	+1.25
	190	0.543	0.003	191.5	+0.79

The Ringbom method of calculating the optimum concentration range (45) was carried out on all the Beer's Law curves and the results are given in Table VI. The molar absorptivities for each sugar investigated are listed in Table VII. The molar absorptivities were calculated from the following equation:

$$\varepsilon = \frac{A}{bc}$$

where ε = molar absorptivity in liter $mole^{-1}$ cm^{-1}

A = absorbance (log Io/I)

b = optical path length in cm.

c = concentration in moles per liter.

TABLE VI

OPTIMUM CONCENTRATION RANGE AS CALCULATED BY THE RINGBOM METHOD

Beer's law plot	Optimum concentration range (µg/ml)
Xylose	8 - 50
Ribose	15 - 85
Glucose	20 - 80
Fructose	15 - 80
Mannose	40 -150
Galactose	40 -140
Glucuronic acid	50 -200
Galacturonic acid	60 -250

TABLE VII

MOLAR ABSORPTIVITIES OF SUGARS USING

THE MODIFIED PROCEDURE

Sugar	Absorption maxima (mμ)	Molar absorptivity (ε x 10-3)
Xylose	390	28.3
Ribose	390	14.8
Glucose	410	20.2
Fructose	410	21.5
Mannose	410	7.64
Galactose	410	9.35
Glucuronic acid	390	6.46
Galacturonic acid	395	5.59

B. Alpha-Substituted Cysteines as Colour Reagents

1) Effect of Alpha-substituted Cysteines

Alpha-substituted cysteines were used as colour reagents instead of cysteine hydrochloride. Spectral studies were made employing α-methyl, α-n-propyl, α-n-butyl, and α-phenyl cysteines (Figures 6-9). The effect of α-substituted cysteines, as colour reagents, is compared with cysteine hydrochloride with respect to absorption maximum, time of standing after the addition of colour reagent, absorbance, and stability of reaction products (Table VIII).

2) Determination of Sugars with α-Methyl Cysteine

A linear relationship of the absorbance to the concentration of sugar was observed using α-methyl cysteine as the colour reagent. The sugars investigated were xylose, glucose, and glucuronic acid. The results are given in Table IX and the Beer's law plots are illustrated in Figure 10.

The molar absorptivities of the three sugars investigated employing α-substituted cysteines as colour reagents are compared to those with cysteine hydrochloride (Table XI).

FIGURE 6

ABSORPTION SPECTRA OF SUGARS WITH

∝-METHYL CYSTEINE AS COLOUR REAGENT

Legend

Spectra of various sugars using ∝-methyl cysteine as colour reagent: Curve 1, 80 µg xylose; Curve 2, 100 µg glucuronic acid; Curve 3, 80 µg glucose.

FIGURE 6

ABSORPTION SPECTRA OF SUGARS WITH

∝-METHYL CYSTEINE AS COLOUR REAGENT

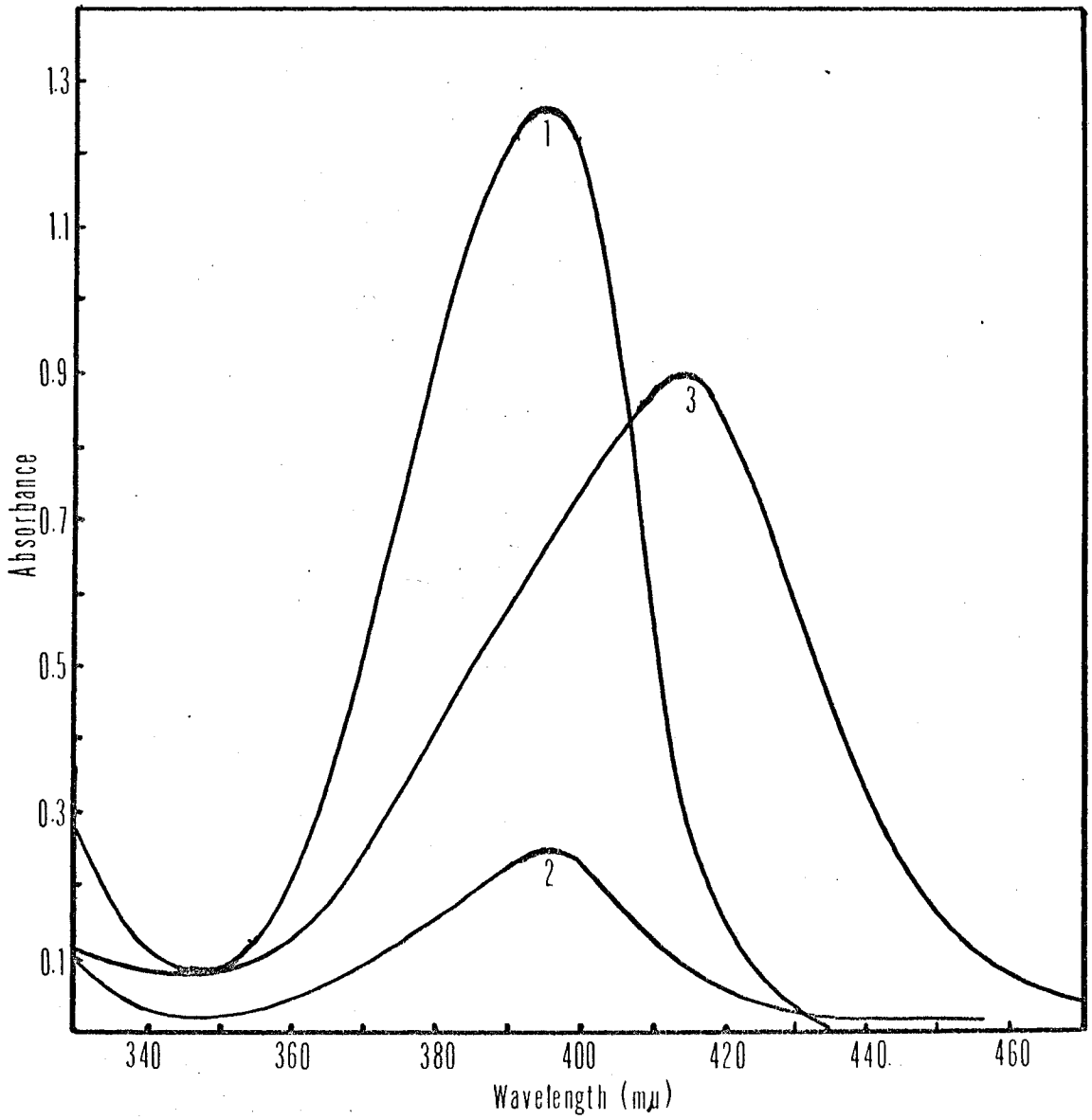


FIGURE 7

ABSORPTION SPECTRA OF SUGARS WITH ∝-n-PROPYL CYSTEINE AS COLOUR REAGENT

Legend

Spectra of various sugars employing ∝-n-propyl cysteine as colour reagent: Curve 1, 80 µg xylose; Curve 2, 80 µg glucose; Curve 3, 100 µg glucuronic acid.

FIGURE 7

ABSORPTION SPECTRA OF SUGARS WITH

∝-n-PROPYL CYSTEINE AS COLOUR REAGENT

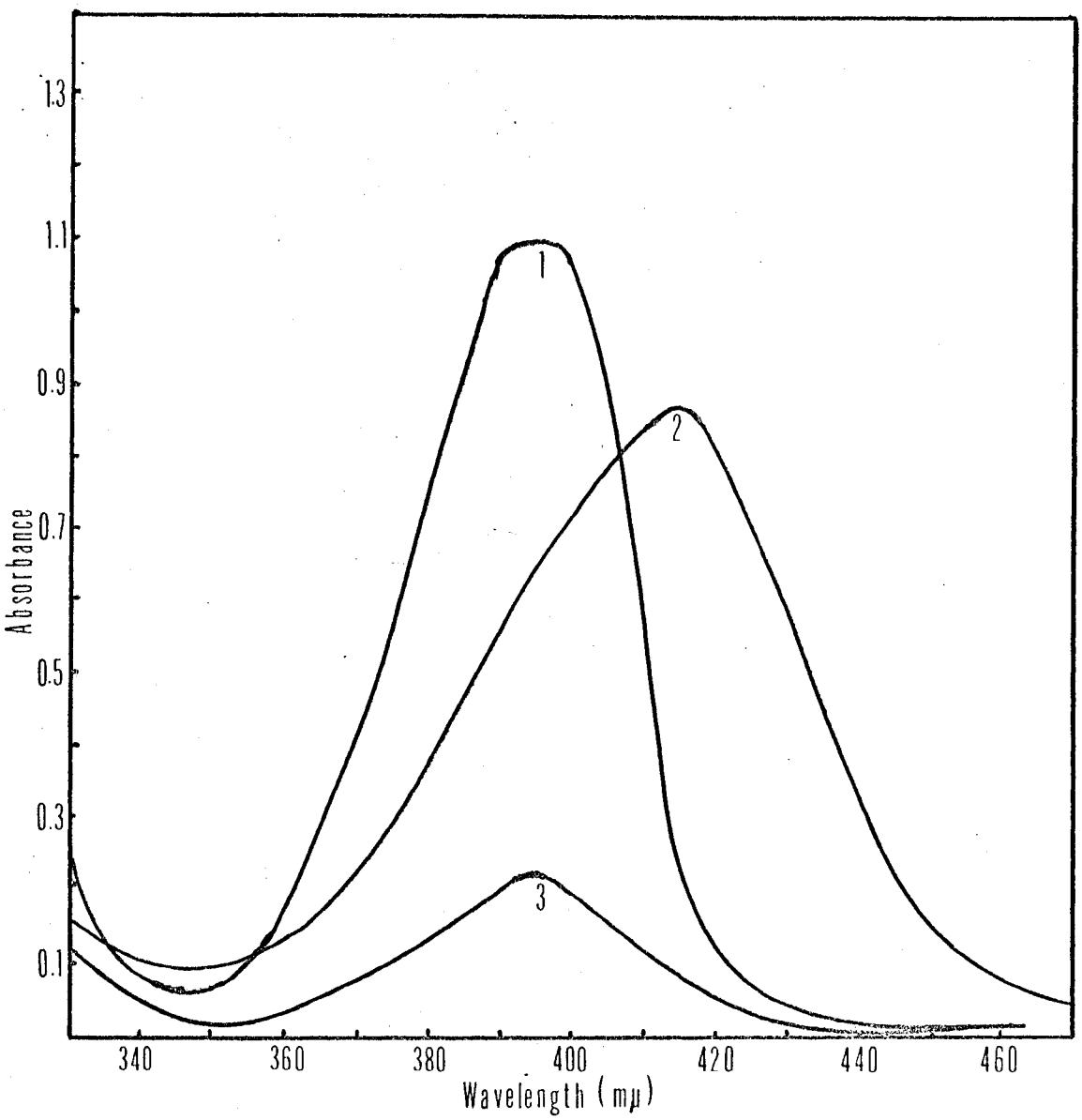


FIGURE 8

ABSORPTION SPECTRA OF SUGARS

WITH ∝-n-BUTYL CYSTEINE

Legend

Spectra of various sugars employing ∝-n-butyl cysteine as colour reagent: Curve 1, 80 µg xylose; Curve 2, 100 µg glucose; Curve 3, 100 µg glucuronic acid.

FIGURE 8

ABSORPTION SPECTRA OF SUGARS WITH

∝-n-BUTYL CYSTEINE

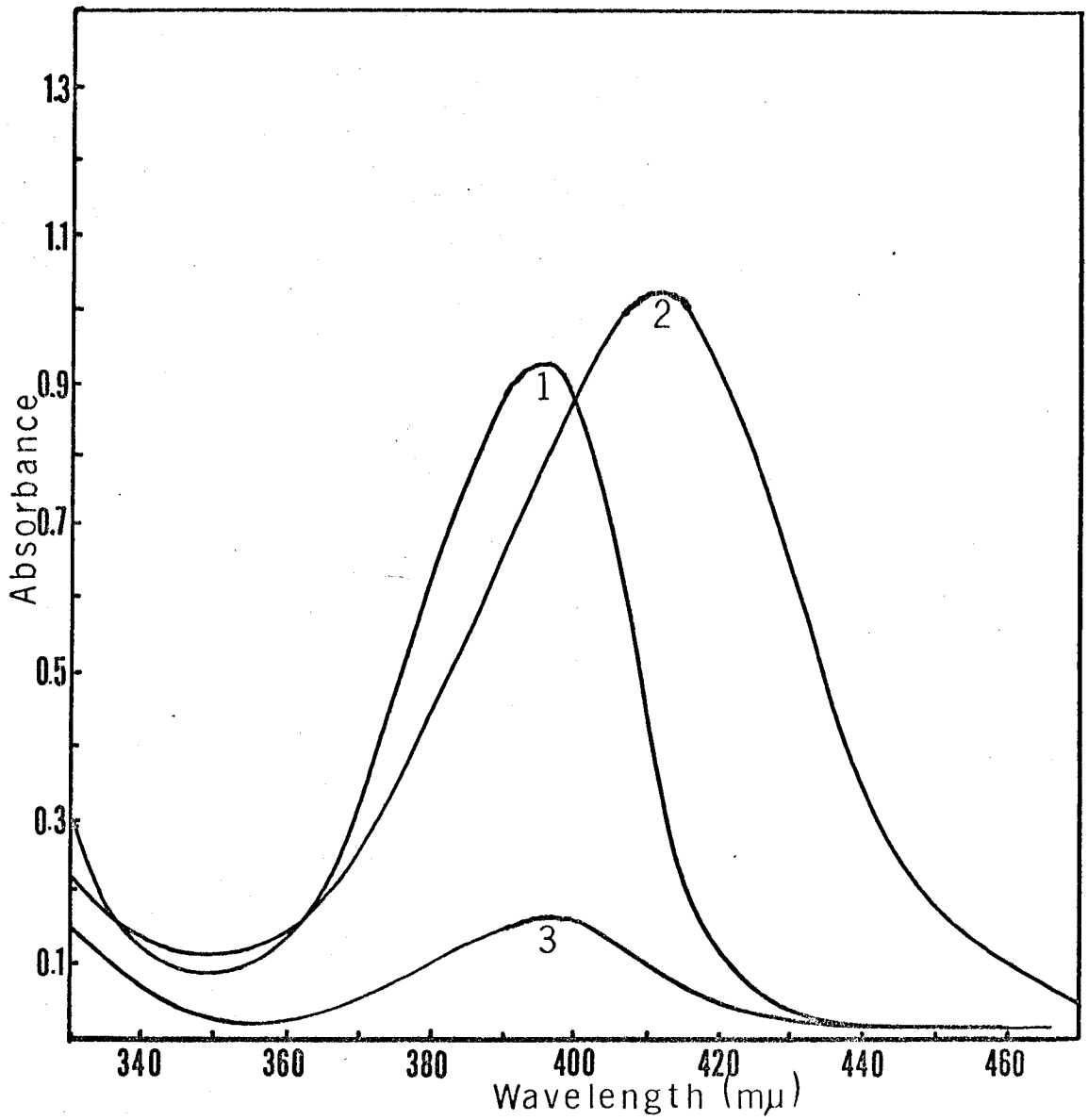


FIGURE 9

ABSORPTION SPECTRA OF SUGARS WITH

α-PHENYL CYSTEINE

Legend

Spectra of various sugars employing α-phenyl cysteine as the colour reagent: Curve 1, 80 µg xylose; Curve 2, 80 µg glucose; Curve 3, 100 µg glucuronic acid.

FIGURE 9

ABSORPTION SPECTRA OF SUGARS WITH

∝-PHENYL CYSTEINE

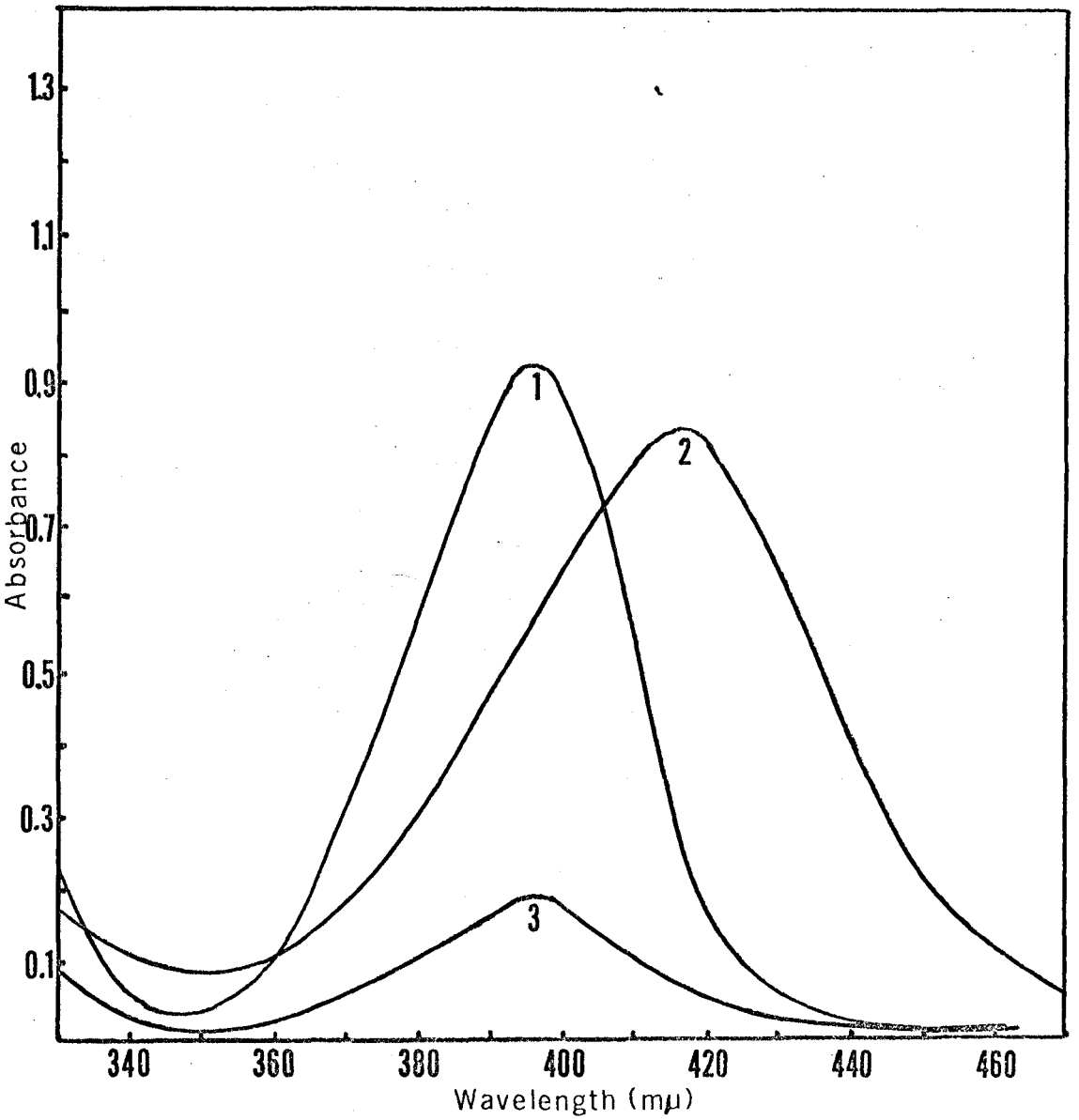


TABLE VIII

EFFECT OF ALPHA-SUBSTITUTED CYSTEINES

Color reagent	Sugar	Concentration (µg)	Absorption maxima (mµ)	Time of standing (min)	Absorbance	Stability (min)
Cysteine -HCl	Xylose	80	390	35	1.443	30
	Glucose	80	408-410	15	0.849	60
	Glucuronic acid	100	390	20-30	0.295	30
α-Methyl-cysteine	Xylose	80	395	25	1.485	45
	Glucose	80	415	15	.847	60
	Glucuronic acid	100	395	30	.286	45
α-n-Propyl-cysteine	Xylose	80	395	30	1.470	40
	Glucose	80	415	15	.845	60
	Glucuronic acid	100	395	25	.286	45
α-n-Butyl-cysteine	Xylose	80	395	35	1.460	45
	Glucose	100	410	15	1.10	60
	Glucuronic acid	100	395	35	.282	45
α-Phenyl-cysteine	Xylose	80	395	30	1.460	35
	Glucose	80	417	15	.845	60
	Glucuronic acid	100	395	25	.254	35

TABLE IX

BEER'S LAW STUDY OF SUGARS

USING α-METHYL CYSTEINE AS COLOUR REAGENT

Sugar	Concentration (μg/ml)	Absorbance	*Average deviation (±)
Xylose	16	.317	.004
	32	.643	.001
	40	.786	.002
	56	1.082	.001
	64	1.243	.002
	72	1.372	.004
	80	1.485	.002
Glucose	15	.158	.001
	30	.312	.003
	45	.476	.008
	60	.641	.005
	70	.742	.001
	80	.847	.005
	90	.963	.001
	100	1.060	.008
Glucuronic acid	40	.127	.002
	60	.180	.003
	100	.286	.003
	120	.345	.002

. . continued

Table IX(continued)

140	.407	.002
160	.465	.007
180	.524	.002
200	.580	.006

* Average deviation in absorbance units

FIGURE 10

BEER'S LAW PLOTS EMPLOYING ∝-METHYL CYSTEINE AS COLOUR REAGENT

Legend

Linear response of absorbance to amount of sugar in 1 ml sample: Curve 1, xylose, 395 mμ; Curve 2, glucose, 415 mμ; Curve 3, glucuronic acid, 395 mμ.

FIGURE 10

BEER'S LAW PLOTS EMPLOYING ∝-METHYL CYSTEINE AS COLOUR REAGENT

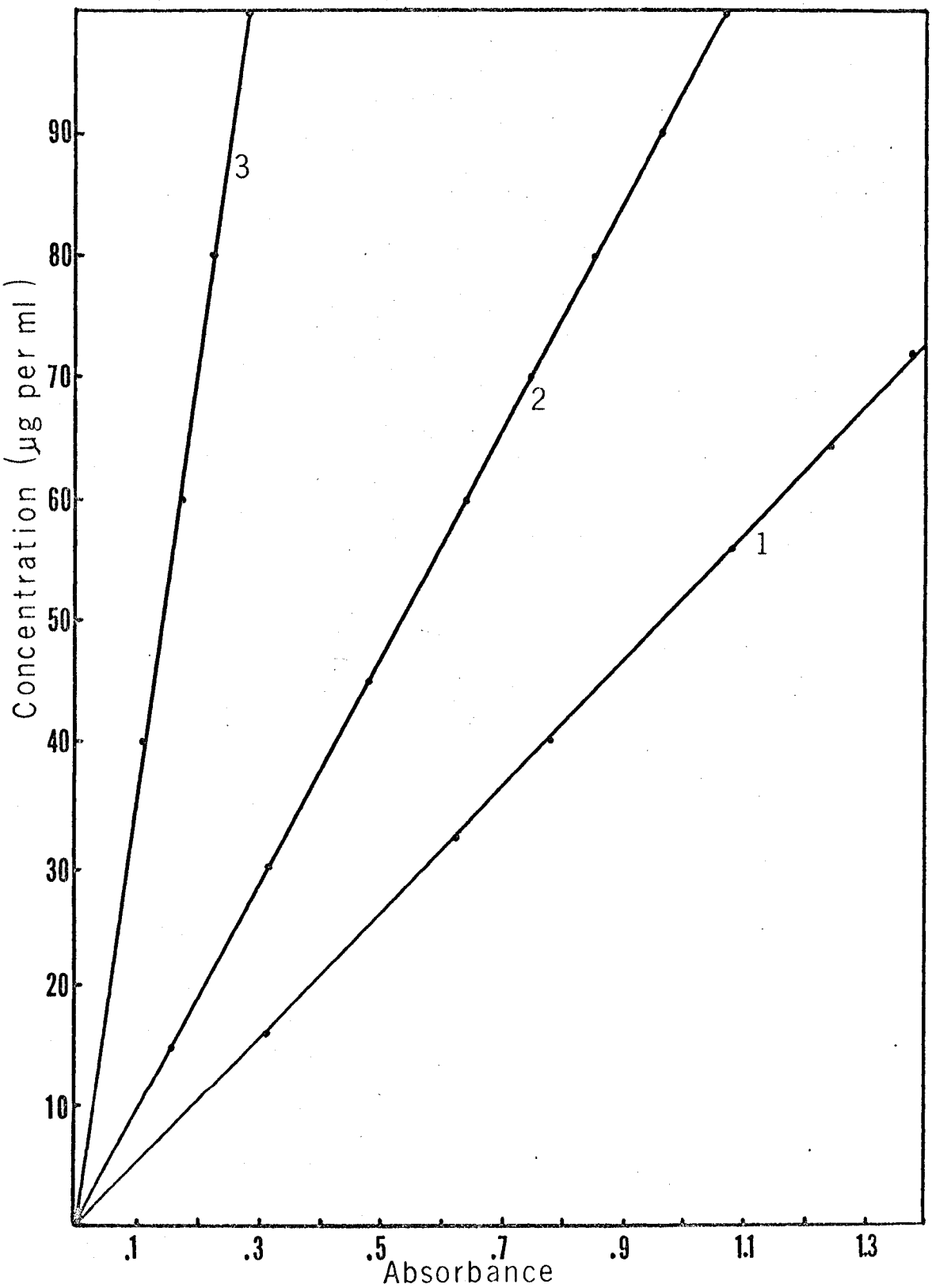


TABLE X

ACCURACY AND REPRODUCIBILITY OF BEER'S LAW PLOTS

USING α-METHYL CYSTEINE AS COLOUR REAGENT

Sugar	Amount added (µg)	Absorbance (average)	*Deviation (±)	Amount recovered (µg)	% error
Xylose	8	0.156	.002	8	0.00
	24	0.473	.001	24.4	+1.67
	48	0.939	.002	48.2	+0.42
Glucose	25	0.252	.002	24	-4.00
	50	0.535	.002	50.5	+1.00
	75	0.793	.003	75.3	+0.40
Glucuronic acid	80	0.225	.003	78	-0.25
	130	0.376	.004	130	0.00
	190	0.550	.004	190	0.00

* Deviation in absorbance units

TABLE XI

COMPARISON OF MOLAR ABSORPTIVITIES WITH VARIOUS COLOUR REAGENTS

Colour Reagent	Sugar	Absorption maxima (mμ)	Molar Absorptivity ($\varepsilon \times 10^{-3}$)
Cysteine -HCl	Xylose	390	28.3
	Glucose	410	20.2
	Glucuronic acid	390	6.46
α-Methyl cysteine	Xylose	395	29.6
	Glucose	415	19.5
	Glucuronic acid	395	5.69
α-n-Propyl cysteine	Xylose	395	27.8
	Glucose	415	19.5
	Glucuronic acid	395	5.69
α-n-Butyl cysteine	Xylose	395	27.6
	Glucose	410	20.0
	Glucuronic acid	395	5.56
α-Phenyl cysteine	Xylose	395	27.6
	Glucose	417	19.5
	Glucuronic acid	395	4.98

CHAPTER IV

DISCUSSION

A. Modified Procedure with Cysteine Hydrochloride

The variation of wavelength, absorbance, and stability of reaction products after the addition of colour reagent with respect to various concentrations of sulfuric acid and volumes of acid mixture is shown in Table I. The absorption maxima for all the different ratios of water to sulfuric acid occurred between 400 and 415 mu. It can also be seen that there was an increase in absorbance with respect to increasing the concentration of sulfuric acid. However, at low volumes of sulfuric acid mixture, the stability decreased with respect to time. Dische also observed this slow decrease of absorbance at 415 mu (24). This decrease of absorbance has been explained by Dische and others as the result of the formation of intermediate reaction products other than furfural and its derivatives (24,31,33). In most of Dische's work, the procedure involves a 1:4 to 1:6 ratio of water to sulfuric acid and the volume of the acid mixtures varies from 4.5 to 6 ml (24-28,30). Table I indicates, however, that by increasing the volume of the mixture of H_2O:H_2SO_4, at any ratio, the stability of the reaction products increases with, in some cases, a loss in absorbance. The best combination of stability, speed, and absorbance was achieved with a 1:7 ratio of H_2O:H_2SO_4

and at a volume of 9 ml.

The next most important variable in determining the intensity and stability of the reaction products is the time of heating. The time of heating determines the final concentration of the primary reaction products which consist of furfural and its derivatives (33). The time of heating for pentoses and hexuronic acids which gave rise to maximum absorbance was 15 min (Table II). This particular time of heating is not critical since heating for 10 or 20 minutes at 99-100°C did not produce any significant change in absorbance readings. Heating above 20 min did, however, result in a decrease in absorbance. Therefore, the time of heating chosen for pentoses and hexuronic acids was 15 min.

The time of heating for hexoses is very critical. The optimum time of heating for hexoses is exactly 3 min. This is in complete agreement with Dische's procedure (24,25). When glucose and fructose were heated for more than three min in the boiling water bath a pink colour would develop. This phenomenon was also observed by others (1,33,46). Love (33) attributed the formation of this pink compound to condensation reactions between $-CH_2OH$ groups of the sugar and 5-hydroxymethyl-2-furfuraldehyde. Therefore, the optimum time of heating for pentoses and hexuronic acids which gave rise to maximum absorbance was 15 min and for hexoses 3 min (Table II).

The absorption spectra for the various sugars illustrate that for sugars within the same category the absorption curves are almost symmetrical with respect to the maximum and differ only in height (Figures 1-3). Simultaneous spectrophotometric analysis of a two component system proved unsuccessful (42). This should be expected since the absorption curves overlap considerably. The absorption maxima for pentoses and hexuronic acids occur at 390 mμ and for hexoses at 410 mμ.

The stability of the reaction products is illustrated in Fig. 4. The reaction products for the various sugars studied displayed an initial increase in absorbance reaching a maximum after a certain period of time. For hexoses this maximum was reached after 15 min and for pentoses and hexuronic acids after 30-35 min. The stability of the reaction products at this maximum is maintained for one-half hour to one hour for pentoses and hexoses, respectively. However, readings should be taken at approximately the same time in order to be consistent with all samples.

Dische investigated the stability of most of the sugars studied in this laboratory and for the determination of pentoses, the procedure requires two hours standing before the addition of cysteine (28). Figure 4 indicates that in the present work stability was reached 30-35 min after the immediate addition of cysteine hydrochloride with respect to pentose and hexuronic acid determinations.

In the determination of hexoses, the maximum was reached after 15 min compared to 90-120 min in Dische's reaction (30). Although most of the experimental conditions with respect to time of heating and absorption maxima were not changed significantly with respect to Dische's reaction, the effect of different acid ratio and volume resulted in better stability, speed, and absorbance. The time required to complete one analysis was about one hour for pentoses and hexuronic acids and 30 min for hexoses.

All sugars tested with the modified procedure, as outlined in Table III, obeyed Beer's law with cysteine hydrochloride as the colour reagent (Table IV). Typical Beer's law curves are shown in Figure 5. The reproducibility and accuracy of these Beer's law plots for the different sugars are illustrated in Table V.

Dische was concerned with the determination of carbohydrates in biological materials. The qualitative tests depend on the extent of colour formation over certain periods of time (1,25,28,48,49). The quantitative analysis relied on the difference of optical densities at two or more wavelengths chosen in such a way that the difference of optical densities at these wavelengths were positive for one class and zero or negative for all other classes of sugar in solution (27). The accuracy of the modified procedures given above and Dische's procedure are difficult to compare since the procedures described in this study

were aimed at the determination of isolated sugars or single sugars in a mixture of non-sugars. The accuracy of the modified procedure was ± 5% as shown in two cases in Table V. However, the relative error for most sugar concentrations was about ± 2%.

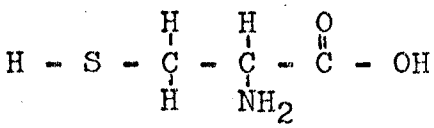
Previous studies show that SH compounds have been used for the qualitative determination of hexuronic acids (28,49). The present work demonstrates that glucuronic acid and galacturonic acid can be determined quantitatively by utilizing the modified procedure. Many procedures have been established to determine glucuronic acid (50). The carbazole-sulfuric acid reaction outlined by Dische (51) is suitable for 5-100 µg glucuronic acid per milliliter and the time required for a complete analysis is approximately three hours. The optimum concentration range as calculated by the Ringbom method for the modified procedure given in this thesis is 50-200 µg glucuronic acid per milliliter (Table VI). The time required for a complete analysis is approximately one hour. Therefore, the modified procedure compares quite well with existing methods for the determination of hexuronic acids and it offers the advantages of speed and a wider concentration range.

The modified procedure proposed for the determination of carbohydrates in simple solutions has the advantages of speed, greater stability, and accuracy. The optimum concentration range for most of the sugars is fairly wide

as shown in Table VI. One could go to lower sugar levels by scaling down the procedure but maintaining the exact ratios of reagents. The overall accuracy is approximately ± 2%.

B. Alpha Substituted Cysteines as Colour Reagents

Cysteine has three functional groups: the sulfhydryl group (-SH), the carboxyl group (-COOH), and the amino group ($-NH_2$) as shown by the following structure:



Structure of Cysteine

Thus if substitution could be effected in the alpha position it could have an influence on the reactivity of these functional groups. Therefore, it was of interest to us to investigate the effect of α-substituted cysteines, as colour reagents, on the absorption maxima, absorbancy, and stability of reaction products.

The absorption spectra employing α-substituted cysteines as colour reagents are quite similar to the absorption spectra involving cysteine hydrochloride (Figures 6-9). The absorption maxima shifted approximately 5 mμ, in most cases, with the greatest shift occurring with α-methyl cysteine. This shift, however, is not significant enough to permit the simultaneous determination of two component systems.

The effect of α-substituted cysteines on colour development, time of standing and stability of the reaction products is given in Table VIII. The table illustrates that the time of standing after the addition of colour reagents was reduced, and that the maxima were maintained for longer periods of time. The largest change in absorbancy occurred with α-methyl cysteine as colour reagent. Comparison of the molar absorptivities (Table XI) with the various colour reagents shows that, in some cases, the α-substituted cysteines are more sensitive towards specific sugars. This is particularly true for α-methyl cysteine. Therefore, if one desired to determine xylose in a pure system the best colour reagent to use would be α-methyl cysteine.

Beer's law was obeyed for the three sugars studied using α-methyl cysteine as colour reagent (Table IX). The Beer's law plots are illustrated in Figure 10. The accuracy was within $\pm$ 2% in most cases and $\pm$ 4% in one instance (Table X). This compares quite well with the accuracy obtained with cysteine hydrochloride. The only disadvantage in utilizing α-substituted cysteines as colour reagents is that they are not commercially available. However, it is definitely apparent that α-substituted cysteines perform as well as cysteine hydrochloride as colour reagents and that in specific cases result in better stability and greater sensitivity.

CHAPTER V

SUMMARY AND CONCLUSIONS

Cysteine hydrochloride was first described by Dische as a colour reagent for the determination of carbohydrates. This reagent has been employed most often as a qualitative test for the identification of sugars. This is a direct consequence of the poor stability of the reaction products formed in sulfuric acid. In this study we have modified the procedure in order to employ the cysteine-sulfuric acid reagent for the quantitative determination of sugars in pure solutions. The modified procedure utilizes a 9-ml volume of H_2O:H_2SO_4 at a ratio of 1:7 H_2O:H_2SO_4 which results in a rapid, non-specific method for the determination of pentoses, hexoses, and hexuronic acids. It was observed that α-substituted cysteines perform as well as cysteine hydrochloride as colour reagents and that in specific cases prove to be more sensitive towards particular sugars. The modified procedure with either cysteine hydrochloride or α-substituted cysteines results in better stability, speed, and absorbancy. The accuracy of the modified procedure is about $\pm$ 2% in the optimum concentration range and the time required for a complete analysis is about one hour for pentoses and hexuronic acids and thirty minutes for hexoses.

This study clarifies the accuracy and reproducibility of the cysteine-sulfuric acid reagent which seems to be

neglected in previous work. The modified procedure which is simple, rapid, and accurate can easily be adapted to the determination of carbohydrates after separation by partition chromatography.

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