Modification of the Anthrone, Carbazole, and Orcinol Reactions for Quantitation of Monosaccharides[1]

W. H. KUNERTH[2] and V. L. YOUNGS[3]

ABSTRACT

The anthrone, carbazole, and orcinol reactions used for quantitation of hexoses, uronic acids, and pentoses, respectively, in Southgate's method for the analysis of unavailable carbohydrates (fiber) were modified to improve specificity by measuring the absorbance at two wavelengths and by using multiple linear regression techniques to correct for remaining interferences. The results of the anthrone reaction were used to correct for hexose interferences in the uronic acid and pentose determinations, and the results of the carbazole reaction were used to correct for uronic acid interferences in the pentose determination.

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Researchers concerned with human nutrition are especially interested in methods that involve fractionation of the components of dietary fiber into specific groups or classes of polysaccharides. When they want detailed information concerning water-soluble and water-insoluble components, researchers often use Southgate's method for unavailable carbohydrates (Southgate 1981).

One problem with this method is the measurement of hexoses, pentoses, and uronic acids using the anthrone, orcinol, and carbazole reactions, respectively. Pentoses and uronic acids interfere with hexose determination, neutral sugars interfere with uronic acid determination, and hexoses and uronic acids interfere with pentose determination. Laine et al (1981) reported that, for the anthrone reaction, the interference caused by xylose was 10%, and for the carbazole reaction, the interference caused by glucose was 16%. For the orcinol reaction, the interferences caused by glucose and glucuronic acid were 4.6 and 49.4%, respectively. Southgate corrected for hexoses when measuring pentoses but did not suggest further corrections. As an alternative to the colorimetric determinations, several researchers use gas-liquid chromatography (GLC) and high-performance liquid chromatography (HPLC) for quantitation of neutral sugars (Englyst 1981, Laine et al 1981, Schweizer and Wursch 1981). However, GLC and HPLC methods are less desirable for analyzing large numbers of samples because so much time is required for sample preparation and analysis.

For part of an investigation into the effects of variety and growing year on the fibrous constituents of durum bran and whole meal, we needed to improve the specificity of the colorimetric methods used for quantitating hexoses, pentoses, and uronic acids. We report modifications to the anthrone, carbazole, and orcinol methods.

MATERIALS AND METHODS

Quantitation of Hexoses

The procedure we used was a modification of the method devised by Roe (1955) and described by Southgate (1981).

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[2]Graduate research assistant, NDSU. Present address: research chemist, USDA, ARS, Hard Red Spring and Durum Wheat Quality Lab, Fargo, ND.

[3]Research leader, USDA, ARS, Hard Red Spring and Durum Wheat Quality Lab, Fargo, ND.

Anthrone reagent. The reagent was prepared by adding 340 ml of water to 660 ml of H_2SO_4 and by then adding 10 g of thiourea and 0.5 g of anthrone (recrystalized from ethanol) to this solution. Although the reagent was said to be stable for two weeks when stored at 4° C, it was prepared either on the day of analysis or one day preceding it.

Standards. A stock solution of 1 mg/ml of glucose in aqueous saturated benzoic acid (SBA) was prepared. Dilutions of the stock solution were made with SBA to give the following solutions: 0 μg/ml, 50 μg/ml, 200 μg/ml, and 250 μg/ml. Each time a set of samples was analyzed, the four standard solutions were measured in duplicate to establish an eight-point standard curve.

Procedure. Two milliliters of the solution to be analyzed was pipetted into a clean, dry test tube. Ten milliliters of anthrone reagent was added to the tube, which was capped and placed in a room-temperature water bath to bring the tube to an equilibrium temperature. Then the tube was heated for 15 min in a vigorously boiling water bath. The tube was removed and cooled in a room-temperature water bath in the dark for 20–30 min. The absorbance was measured at 620 and 575 nm against a procedure blank.

Calculations. The concentration of hexoses in the test sample was calculated using equation 1, in which the values for slope and intercept were calculated from the standard curve data using regression analysis with HABS as the dependent variable and hexose concentration as the independent variable. The correlation coefficient (r) for the regression was always greater than 0.995. Each sample was determined in duplicate and the mean value reported. This value was also used for calculating the concentration of uronic acids and pentoses present in the sample.

$$HEX = (HABS - I)/S \quad (1)$$

where HEX = the concentration (μg/ml) of hexoses in the test sample; HABS = absorbance at 620 nm minus the absorbance at 575nm; I = intercept; and S = slope.

Measurement of Uronic Acids

The procedure we used was a modification of the method described by Bitter and Muir (1962) and by Southgate (1981).

Carbazole reagents. The carbazole reagents consisted of a borax solution containing 9.52 g of $Na_2B_4O_7 \cdot 10H_2O$/L and a solution containing 0.125% carbazole in ethanol.

Standards. A series of eight standard solutions, each containing a mixture of monosaccharides, was prepared from 1 mg/ml stock solutions of galacturonic acid, xylose, arabinose, glucose, and galactose in SBA. The ranges of concentrations used were as follows: 0–150 μg/ml total pentose, 0–175 μg/ml total hexose, and 0–50 μg/ml galacturonic acid. Total hexose was considered the sum of glucose and galactose concentrations, and total pentose as the sum of arabinose and xylose concentrations.

Procedure. For each sample being assayed, two test tubes containing 5 ml of borax solution were cooled in an ice bath. One milliliter of sample was added to each tube, the tubes were capped, and the solutions were mixed thoroughly but gently. The tubes were kept in the ice bath to allow for temperature equilibrium. Next, the tubes were placed in a boiling water bath for 10 min and then were cooled in a room-temperature water bath. Carbazole solution (0.2 ml) was added to one of the tubes, and both tubes were heated in a boiling-water bath for 15 min. After cooling in a room-temperature water bath, the absorbance of the tube containing the carbazole was measured at 530 nm and 660 nm against the tube without carbazole.

It was essential that all heating and cooling of the test sample be done simultaneously with the standards. A slight difference in time or temperature affected the results significantly.

Calculations. The concentration of uronic acids in the test sample was calculated using equation 2, in which the values for slope and intercept were calculated from the standard curve data using regression analysis with UABS as the dependent variable and uronic acid and hexose concentrations as independent variables. The correlation coefficient (r) for the regression was always 0.990 or greater. The sample was assayed in duplicate, and the mean value was reported. This value was also used in calculating the concentration of pentoses in the sample.

$$UA = (UABS - I - (HEX \times S1))/S2 \quad (2)$$

where UA = concentration of uronic acids in test sample in μg/ml; UABS = absorbance at 530 nm minus the absorbance at 660 nm; I = intercept; HEX = concentration of hexose in the sample determined by the anthrone reaction; S1 = contribution to the slope due to hexoses; and S2 = contribution to the slope due to uronic acids.

Measurement of Pentoses

The procedure used was a modification of the method originally proposed by Albaum and Umbreit (1947) and used by Southgate (1981).

Orcinol reagent. The orcinol reagents consisted of 0.1% $FeCl_3$ in 12.1 *N* HCl and 10% orcinol in 95% ethanol. Immediately before use, the orcinol reagent was prepared by mixing 25 parts of 10% orcinol solution with 250 parts of 0.1% $FeCl_3$ solution.

Standards. A series of eight standard solutions, each containing a mixture of monosaccharides, was prepared from 1 mg/ml solutions of xylose, glucose, galactose, and galacturonic acid in SBA. The ranges of concentration were 0–20 μg/ml xylose, 0–40 μg/ml total hexose, and 0–10 μg/ml galacturonic acid. Total hexose was considered as the sum of glucose and galactose concentrations.

Procedure. Up to 3 ml of the solution to be tested was measured into a test tube, and the volume was adjusted to 3 ml. Four milliliters of orcinol reagent was added to the tube, which was capped and mixed by shaking. Then the solution was heated in a boiling-water bath for 45 min, cooled in a room-temperature water bath, and the absorbance measured at 670 and 600 nm against a procedure blank.

Calculations. The concentration of pentoses in the test sample was calculated using equation 3, in which the values for slope and intercept were calculated from the standard curve data using regression analysis with PABS as the dependent variable and pentose, hexose, and uronic acid concentrations as independent variables. The correlation coefficient (r) for the regression was always greater than 0.995. Each sample was determined in duplicate with the mean value reported.

$$PENT = (PABS - I - (HEX \times S1) - (UA \times S2))/S3 \quad (3)$$

PENT = concentration of pentose in test sample in μg/ml; PABS = absorbance at 670 nm minus the absorbance at 600 nm; I = intercept; HEX = concentration of hexoses in the sample determined by the anthrone reaction corrected for dilution; UA = concentration of uronic acids in the sample determined by the carbazole reaction corrected for dilution; S1 = contribution to slope due to hexoses; S2 = contribution to slope due to uronic acids; and S3 = contribution to slope due to pentoses.

Data Manipulations

All calculations and statistical manipulations were done using a system consisting of IBM 4341 and IBM 370/158 computers equipped with the Statistical Analysis Systems (SAS) computer package (Statistical Analysis System 1979).

RESULTS AND DISCUSSION

The anthrone, carbazole, and orcinol reactions as described by Southgate (1981) were evaluated for specificity. With one exception, the results agreed with the interference values reported by Laine et al (1981). For the anthrone reaction, arabinose gave 7.2% of the response of an equal concentration of glucose. Laine et al had reported that arabinose did not interfere. Other interferences include the following: For the anthrone reaction, galacturonic acid gave 10% of the response of glucose; for the carbazole reaction, the responses of arabinose, xylose, and galactose were 5.9%, 7.6%, and 17.1%, respectively, of the response of galacturonic acid; for the orcinol reaction, the interference due to galactose was 11% compared to arabinose.

For the anthrone reaction, specificity was improved by

measuring the absorbance at two wavelengths (620 and 575 nm) instead of the single wavelength (620 nm) specified by Southgate. Figure 1 A and B shows the absorbance curves obtained by treating solutions of glucose, arabinose, xylose, and galacturonic acid with anthrone reagent. The difference in absorbance between the two wavelengths was directly related to hexose concentration. Measurement of the absorbance at two wavelengths allowed for reduction of the interferences caused by arabinose, xylose, and galacturonic acid because the absorbances at 575 and 620 nm were nearly equal for each of the three monosaccharides; thus the absorbance at 620 nm minus the absorbance at 575 nm was nearly zero. The responses of glucose and galactose were also reduced but reduced proportionately less than arabinose, xylose, and galacturonic acid. The interference due to uronic acids was reduced to less than 4.5%, and the effect due to pentoses was statistically nonsignificant ($P = 0.05$)[4] when total pentose concentration was in the range of 0–150 μg/ml. At higher pentose concentrations the presence of pentoses becomes significant. Concentrations of uronic acids, normally found in wheat products, had a minimal effect on hexose measurement.

One concern with the anthrone reaction was that glucose and galactose responded differently. Galactose gave 80% of the response of glucose. Since glucose was the major hexose measured, the standard curve was prepared using glucose alone. Because the response from galactose was less than that from glucose, the use of glucose as a standard resulted in underestimating that portion of hexose present as galactose, but resulted in a more accurate measurement of glucose.

For the carbazole reaction, measurement of the absorbance at two wavelengths (530 and 660 nm) instead of the single wavelength (530 nm) improved specificity. Figure 2A and B shows the absorbance curves obtained by treating solutions of glucose, galactose, arabinose, xylose, and galacturonic acid with carbazole

TABLE I
Standard Additions of Monosaccharides to Water-Insoluble and Cellulose Fractions[a]

Reaction	Carbohydrate Added	Amount Added (μg)	*n*	Average Recovery Percent + Std Dev
Anthrone	Glucose	100	12	101.7 ± 6.1
Carbazole	Galacturonic acid	25	12	98.6 ± 2.8
Orcinol	Xylose	20	12	98.4 ± 3.5

[a] Six additions to both the water-insoluble and cellulose fractions. The water-insoluble (WIS) and cellulose (C) fractions were analyzed before the standard additions, using modified methods. The results were (WIS) 25 μg/ml hexose, 3.7 μg/ml uronic acid, and 48 μg/ml pentose; (C) 65 μg/ml hexose, 0.0 μg/ml uronic acid and 0.5 μg/ml pentose.

[4] P is the probability of rejecting a true hypothesis.

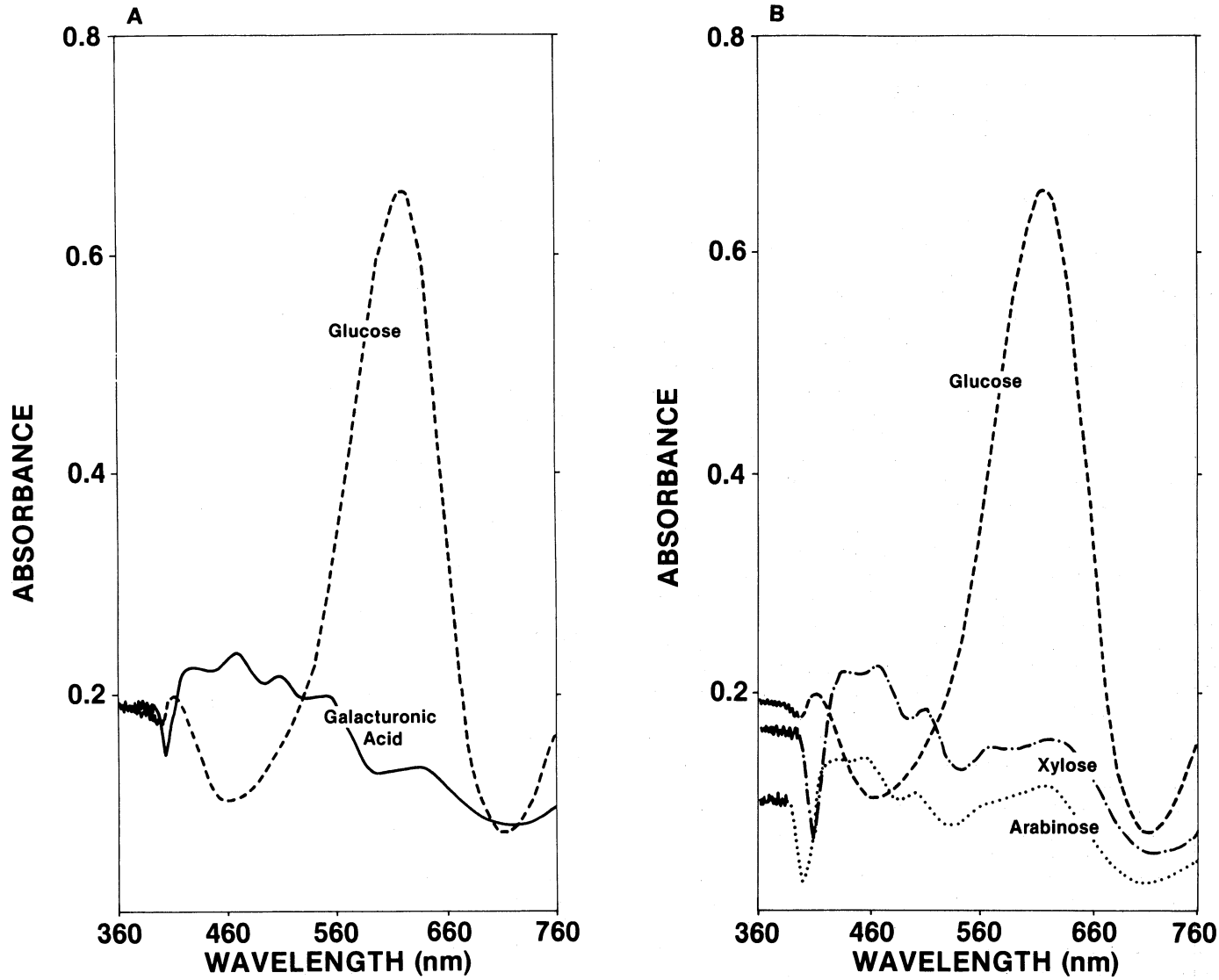

Fig. 1. A, Absorbance scans of 250 μg/ml glucose and 500 μg/ml galacturonic acid solutions subjected to the anthrone reaction. **B,** Absorbance scans of 250 μg/ml glucose, 500 μg/ml xylose, and 500 μg/ml arabinose solutions subjected to the anthrone reaction.

reagent. The difference in absorbance between the two wavelengths was directly related to uronic acid concentration. Galacturonic acid and glucuronic acid gave similar responses. The presence of pentoses was statistically significant, but measurement of the absorbance at two wavelengths decreased the effects of pentoses. The presence of hexoses also had a significant effect. Measurement of the absorbance as the difference between two wavelengths resulted in similar responses from galactose and glucose. Galactose gave approximately 85% of the response of glucose by this measurement (Fig. 2B), and made a single correction for hexoses possible. The value for hexose concentration, determined with the anthrone reaction, was used to correct for the effects due to hexose.

TABLE II
Measurement of Hexoses, Uronic Acids, and Pentoses by the Anthrone, Carbazole, and the Orcinol Reactions and by Modified Versions of the Methods[a]

Standard Solution Concentrations (μg/ml)						Anthrone Reactions[b] (μg/ml hexose)		Carbazole Reactions[b] (μg/ml uronic acid)		Orcinol Reactions[b] (μg/ml pentose)	
Solution	Glu[c]	Gal[c]	GalOH[e]	Xyl[d]	Ara[d]	A	B	A	B	A	B
1	0	0	0	0	0	−4.61	−3.74	1.60	−0.75	−2.07	0.61
2	0	20	0	5	20	13.79	12.93	5.55	0.89	24.26	23.21
3	25	25	50	5	75	52.54	50.97	59.94	49.58	111.88	83.53
4	5	5	40	50	50	18.29	10.16	41.92	37.63	123.16	98.27
5	75	10	30	25	5	86.02	87.87	43.93	28.80	48.10	27.29
6	100	0	20	50	5	103.22	105.52	37.22	19.22	63.57	48.42
7	125	40	10	10	10	158.59	161.27	33.34	9.20	31.87	18.54
8	150	50	25	100	50	208.11	219.23	57.72	24.19	177.04	156.95

[a] Method A = methods as described by Southgate (1981). Method B = methods as described in the present article.
[b] Each value shown is the mean of four analyses.
[c] Hexose.
[d] Pentose.
[e] Galacturonic acid.

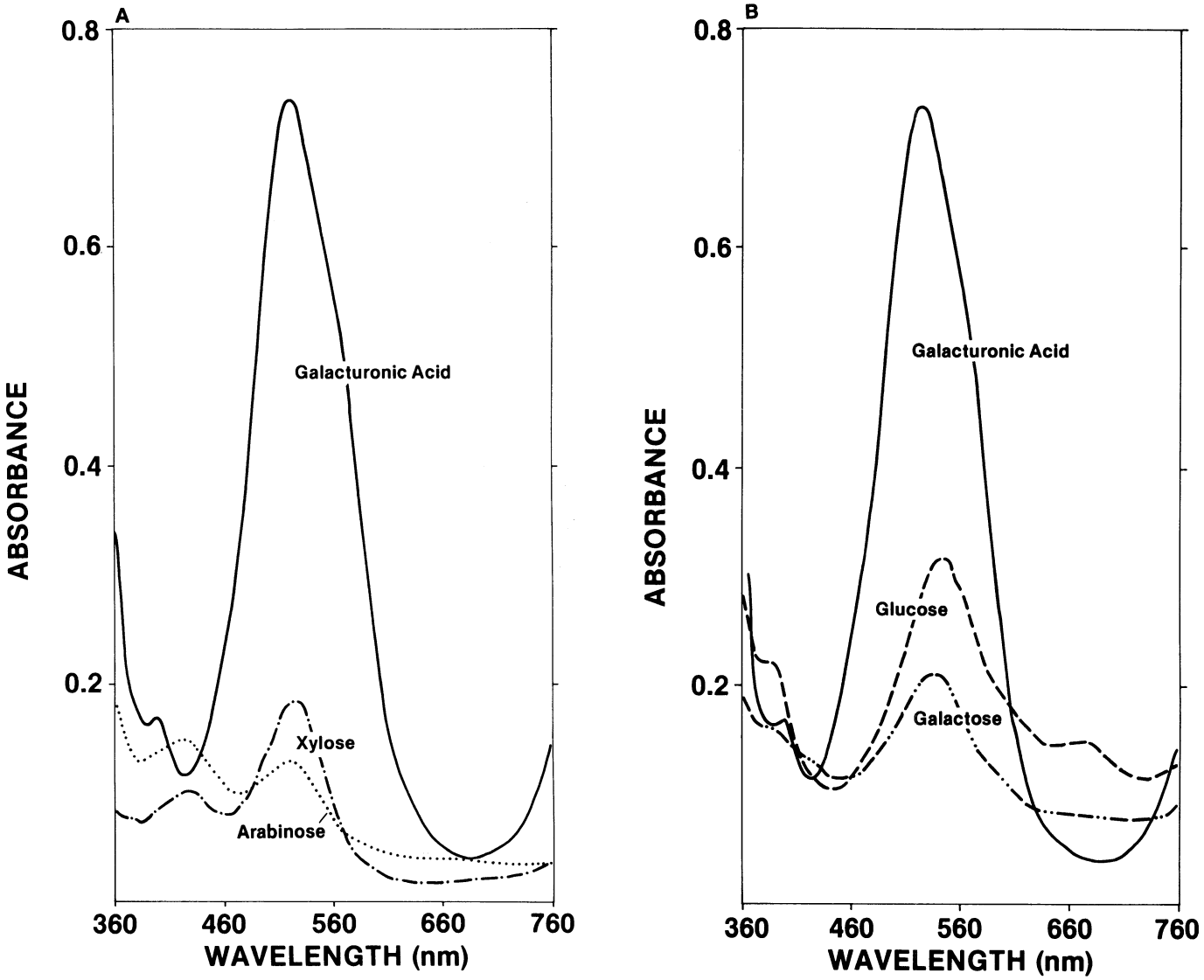

Fig. 2. A, Absorbance scans of 50 μg/ml galacturonic acid, 200 μg/ml xylose, and 200 μg/ml arabinose solutions subjected to the carbazole reaction. **B,** Absorbance scans of 50 μg/ml galacturonic acid, 100 μg/ml glucose, and 100 μg/ml galactose solutions subjected to the carbazole reaction.

Because the effect of xylose was not identical to the effect of arabinose, and the effect of glucose was not identical to that of galactose, the solutions used in preparing the standard curve contained glucose, galactose, arabinose, and xylose in varied concentrations in addition to galacturonic acid. For determination of the effects of pentoses and hexoses, pentoses were considered to be the sum of arabinose and xylose concentrations, and hexoses to be the sum of glucose and galactose concentrations.

Southgate reported that when a large excess of hexoses was present, the carbazole reaction produced a brown color because of the effect of the acid on hexose(s). He indicated that this interference could be decreased by measuring the absorbance of each test solution against a blank containing the test sample, but without carbazole. For this study every sample, including the standards, had the appropriate blank prepared in this fashion.

Dische (1962) suggested that, when measuring pentoses by the orcinol reaction, interferences from hexose could be eliminated by subtracting the absorbance at 600 nm from the absorbance at 670

TABLE III
Examples of Regression Equations Used in Calculating Results in Table II[a]

Determination	Method[b]	Equation[c]
Hexose	A	A620 = 0.01319 + (HEX) (0.00269)
	B	A620−A575 = 0.00268 + (HEX) (0.00082)
Uronic acid	A	A530 = −0.00795 + (UA) (0.01348)
	B	A530−A660 = 0.01602 + (UA) (0.01286) + (HEX) (0.00130)
Pentose	A	A670 = 0.00141 + 0.06672 (PENT)
	B	A670−A600 = −0.00269 + (PENT) (0.04126) + (HEX) (0.00044) + (UA) (0.02330)

[a]Since a new standard curve was prepared for each set of samples analyzed, the regression equations shown here are only one of the four equations of each type used in calculating the values shown in Table II. However, the values are representative of the values normally observed.
[b]Method A = methods as described by Southgate (1981). Method B = methods as described in the present article.
[c]A620 refers to the absorbance at 620 nm; the same pattern is used for describing each of the other absorbance variables. HEX, UA, and PENT refer to hexose, uronic acid, and pentose concentrations, respectively.

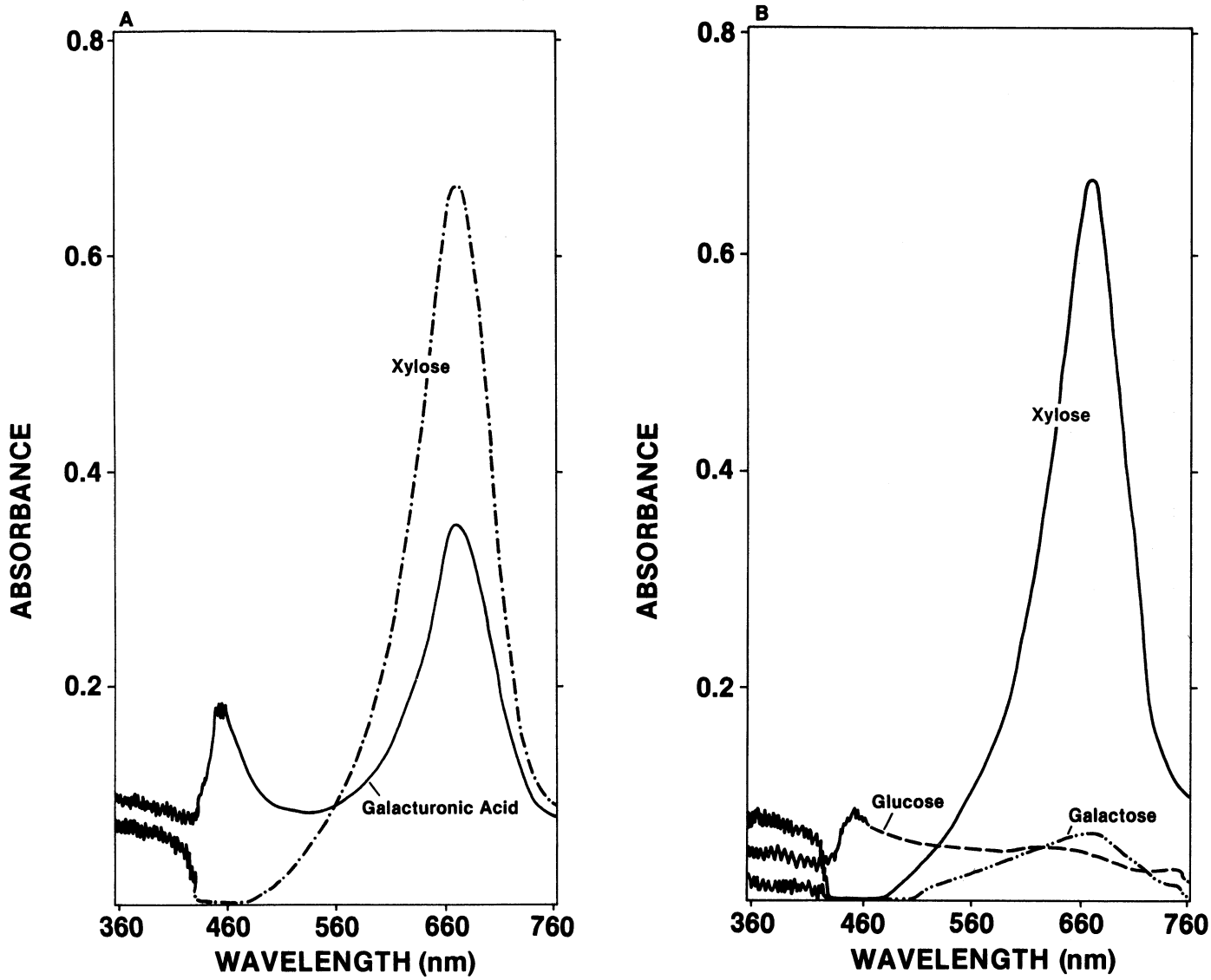

Fig. 3. A, Absorbance scans of 10 μg/ml xylose and 10 μg/ml galacturonic acid solutions subjected to the orcinol reagent. **B,** Absorbance scans of 10 μg/ml xylose, 10 μg/ml glucose, and 10 μg/ml galactose solutions subjected to the orcinol reagent.

nm. Dische's modification, which was used, substantially decreased but did not eliminate the interference due to hexose. The modification had no effect on the interference due to uronic acids. Figure 3 A and B shows the absorbance curves obtained by treating solutions of xylose, glucose, galactose, and galacturonic acid with orcinol reagent. When calculating the concentrations of pentose using the linear model, corrections for the remaining interference due to hexoses and for the interference due to uronic acids were required. The corrections were based on hexose and uronic acid concentrations, determined by using the anthrone and carbazole reactions.

One concern with the orcinol reaction was that glucose and galactose responded differently. Glucose had a stronger absorbance at 600 nm than at 670 nm, but galactose showed a stronger absorbance at 670 nm. This would be a potential source of error when glucose concentration was much higher than galactose, as in the cellulose fraction. The result of this discrepancy would be that the pentose contribution to the cellulose fraction would be slightly underestimated.

Although reproducibilities with the orcinol and anthrone reactions were good, new standard curves were prepared for each set of samples tested. Reproducibility for the carbazole reaction was poor, and it was essential that a new standard curve be prepared each time samples were analyzed.

A series of standard additions was made to solutions containing the water-insoluble and cellulose fractions obtained from the Southgate fractionation of durum whole meal and bran samples. The recoveries of standard additions were measured as hexoses, pentoses, and uronic acids (Table I).

To verify the modifications to the colorimetric methods, a series of standard solutions was analyzed using the procedures described by Southgate (1981) and by the procedures described in Methods. The analyses were conducted by a college sophomore majoring in chemistry who was relatively inexperienced in conducting analyses of this type. The mean results of four sets of analyses are shown in Table II. These results represent the degree of improvement expected when the modified methods are used. In nearly all cases, there were improvements over Southgate's procedures. The most serious exception was in the hexose determination, in which the value obtained for galactose in solution 1 was 6.2% lower than that obtained by the Southgate procedure and in solution 8, in which the value for total hexose was 5.3% larger than the value obtained from Southgate's procedure. This was balanced by the improvement observed in solution 4, in which the value from the modified method was 1.6% above the actual concentration and the value obtained by the Southgate procedure was 82.9% above. The most significant improvements were observed for the uronic acid and pentose determinations. Table III shows examples of the regression equations used in calculating the values shown in Table II.

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