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CEREAL CARBOHYDRATE DETERMINATION

Sulfonated 1-Naphthol and Anthrone Reactions Applied to Sulfuric Acid Extract of Cereals

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Proximate analysis is satisfactory for the estimation of protein, ash, and "crude fat" content of cereals and cereal grains. However, there is need for a simple method for the direct estimation of carbohydrates in the nitrogen-free extract. The sulfonated 1-naphthol reaction (modified Molisch test) not only gives consistent results for the estimation of carbohydrate in the 1.25% sulfuric acid extract, but it also offers a method of estimating pentose in this extract. As the anthrone reaction yields a stable color for hexoses and an unstable color for pentoses, this test can be used as a check for the sulfonated 1-naphthol reaction. These tests can be run on aliquots of the 1.25% sulfuric acid extract when crude fiber is determined.

ONLY THE PROTEIN, ASH, FAT (ether extract), and crude fiber contents are shown by the proximate analysis of cereals. The remainder of the dry material is assumed to be soluble carbohydrate and is called the nitrogen-free extract (NFE). A simple method is needed for determining carbohydrates in this fraction, which may contain some bound water and pentosans which are of very little value in human nutrition.

The absorption band of the colored products from the reaction of sulfonated 1-naphthol and sulfuric acid (modified Molisch test) is more in the violet (*1, 3–5*) for pentoses than for hexoses. Consistent results are obtained for both types of carbohydrates.

The color formed in the anthrone reaction with pentoses fades rapidly (*6–8*), whereas the color with hexoses is comparatively stable.

The objectives of the present work were: to determine the precision of the sulfonated 1-naphthol reaction when it is applied to the sulfuric acid extracts of cereals and cereal products; to study the possibility of using both the anthrone and the sulfonated 1-naphthol reactions for estimating pentoses present in the extract; and to develop a simple rapid procedure for the determination of carbohydrates in the extract, which may be applied when crude fiber is determined.

Methods and Materials

Procedure Extraction Two-hundred milligram samples of ground material were extracted by refluxing with 50 ml. of 1.25% sulfuric acid (1.25 grams per 100 ml.) for 30 minutes. The mixture was cooled, made to 100 ml., and filtered. All extractions were made in triplicate. An aliquot of the filtrate, containing 6 to 8 mg. of carbohydrate, was diluted to 200 ml. These diluted extracts were used for the determinations.

Sulfonated 1-Naphthol Reaction. Five milliliters of concentrated sulfuric acid was rapidly added from a delivery pipet to a test tube (while mixing) containing 1 ml. of sulfonated 1-naphthol reagent (*1*) and 1 ml. of the diluted extract. The time allowed for the pipet to drain was constant (10 seconds) and the hot mixture was vigorously agitated for 15 seconds longer and then placed in a boiling water bath. After a total heating period of 8 minutes the tube was placed in a mixture of ice and water until the spectrophotometric readings were made. When the tubes were cool, the absorbances were measured at 530, 540, 550, 560, 570, and 580 mμ with a Model B Beckman spectrophotometer in 1-cm. cells. The reference mixture contained 1 ml. of distilled water in place of the extract and the standard contained 0.040 mg. of carbohydrate in 1 ml. of water. These were treated the same as the extract.

Anthrone Reaction. Ten milliliters of 0.2% anthrone reagent (0.2 gram of anthrone per 100 ml. of concentrated sulfuric acid) was placed in a 50-ml. Erlenmeyer flask containing 5 ml. of the diluted extract, and the contents of the flask were thoroughly mixed. After standing for 1 hour, the absorbances were measured at 620 and 630 mμ with the Model B Beckman spectrophotometer in 1-cm. cells. The reference mixture contained 5 ml. of distilled water in place of the extract and the standard contained 0.200 mg. of carbohydrate in 5 ml. of water. The standard and the blank (reference mixture) were treated the same as the diluted extract.

Results and Discussion

Estimation of Carbohydrates **Sulfonated 1-Naphthol Reaction.** When this reaction is applied to hexoses, the maximum absorption for the colored products occurs at 573 mμ (Figure 1), whereas the pentoses yield products with the maximum absorption near 552 mμ. Mixtures of pentoses and hexoses yield products with an absorption maximum between these wave lengths. In the present study, mixtures of xylose and glucose were investigated in detail. It was discovered that a given weight of pure glucose and the same weight of glucose-xylose mixtures, containing as much as 14% xylose, yielded colored products with almost the same absorbance at 570 mμ. When the xylose content is increased enough, the peak will rise and the absorbance at 570 mμ will fall. Hence, one can use a glucose standard for estimating carbohydrates when glucose and xylose are the principal sugars present, as long as the xylose content is not too high.

The results for the estimation of the carbohydrates (calculated as monosaccharide) in the sulfuric acid extract are shown in the second column of Table I. The almost theoretical value for corn and wheat starches indicates that starch is nearly 100% recovered. Similar results were obtained (not shown) for mixtures of xylose and glucose.

From the percentage of monosaccharide, it was simple matter to calculate the percentage of polysaccharide extracted by the 1.25% sulfuric acid. The results as shown in the ninth and eleventh columns of Table I (pentose determination is discussed below) indicate that the polysaccharide content of this extract is less than the nitrogen-free extract values. The greatest difference was noted for the sample of oats, where the nitrogen-free extract value was 73.0% while the polysaccharide content in the 1.25% sulfuric acid extract was only 51.2%. Otherwise, the polysaccharide content of the extract seems to approach the nitrogen-free extract value. Moreover, wheat flour yielded an extract in which the polysaccharide content was almost the same as the nitrogen-free extract. Preliminary extraction of fat did (*2*) not seem to affect the value. The nitrogen-free extract values were obtained from Edward R. Binnewies, South Dakota State College, who used the samples as unknowns in his food analysis course.

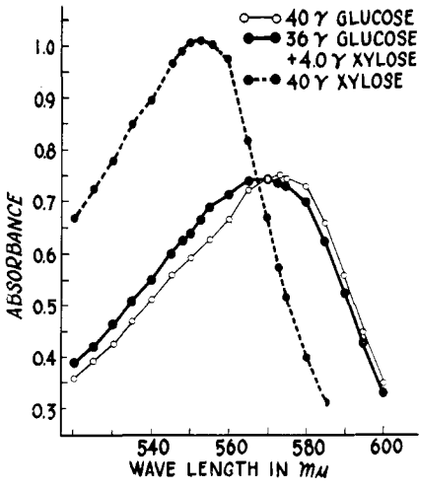

Figure 1. Absorption bands for colored products formed when pure glucose, 90% glucose in glucose-xylose mixture, and pure xylose were treated with sulfonated 1-naphthol

Estimation of Pentose **Sulfonated 1-Naphthol Reaction.** There is a shift in the absorption band (from the red toward the violet) as the pentose content is increased in the hexose-pentose mixtures. When the amount of carbohydrate (glucose and xylose) is kept constant, the absorbance at 580 mμ decreases as the xylose content is increased, whereas at 530, 540, 550, and 560 mμ, the absorbance increases, as illustrated in Figure 1. This shift in the absorption band makes possible the estimation of xylose in the presence of glucose.

In the study of glucose-xylose mixtures the observed absorbance at 580 mμ was changed to unity and the observed absorbances at 530, 540, 550, and 560 mμ were changed proportionally. For example, when the observed absorbance was 0.600 at 580 mμ and 0.420 at 540 mμ, the absorbance of 0.600 was changed to 1.000 and 0.420 was changed to 420/600 or 0.700. These calculated absorbances were then plotted against percentage of xylose (percentage of xylose in the 40 γ of glucose-xylose standard) and nearly straight lines were obtained as illustrated in Figure 2. When the diluted extract was used, the same calculations were made and the percentage of xylose (percentage of extracted carbohydrate) was read from this graph (Figure 2). The results from these readings are shown in column 5 of Table I. As an illustration, when the calculated absorbances were 0.600 at 530 mμ, 0.707 at 540 mμ, 0.825 at 550 mμ, and 0.940 at 560 mμ, the percentage of xylose was read from the graph (Figure 2) as 3.2, 2.8, 3.3, and 3.4. The per-

Table I. Carbohydrate Content of 1.25% Sulfuric Acid Extract of Cereal Grains and Cereal Products

(Standards were run at same time as sample for repeated experiments. Nitrogen-free extract and crude fiber values are taken from results obtained by Edward Binnewies, S. Dakota State College.)

Material Extracted with 1.25% H_2SO_4	Monosaccharide in Total Sample Calcd. as % Glucose (Glucose Standard)			Pentose (Xylose Standard) Calcd. as % of Extracted Carbohydrates				Polysaccharides Extracted, Calcd. as % of Sample, Sulfonated 1-Naphthol			Proximate Analysis	
	Sulfonated 1-Naphthol			Sulfonated 1-Naphthol		Calcd. from Differences between Color Produced by 2 Methods, Glucose Standard						
		Repeated expts.	Anthrone	From standard curves	Repeated expts.		Repeated expts.	Hexosan as glucan	Pentosan as xylan	Total	NFE	Crude fiber
Oats	57.1	59.0	48.9	12.8	13.0	16.5	11.8	44.8	6.4	51.2	73.0	9.3
Rye	80.0	...	74.5	9.7	...	7.9	...	65.0	6.8	71.8	73.9	3.5
Corn	84.6	...	83.0	3.2	...	2.2	...	73.7	2.4	76.1	80.0	2.2
Wheat	78.5	80.7	71.0	7.0	6.8	11.0	6.8	65.7	4.8	70.5	82.6	2.3
Durum wheat	73.4	...	66.5	9.0	...	10.8	...	60.1	5.8	65.9	73.3	2.5
Millet	68.6	70.3	68.8	2.5	2.5	0.0	0.7	60.2	1.5	61.7	70.5	8.8
Barley	70.5	...	66.0	9.8	...	7.3	...	57.2	6.1	63.3	74.5	5.8
Shredies	84.8	...	81.6	4.9	...	4.3	...	72.6	3.7	76.3	80.0	1.4
Soya-wheat	55.2	55.0	50.0	6.7	6.7	10.8	7.8	46.4	3.3	49.7	56.1	2.8
Corn meal	93.2	...	92.6	2.8	...	0.7	...	82.4	2.3	84.7	85.1	2.5
Wheaties	80.5	...	74.6	6.1	...	8.4	...	68.0	4.3	72.3	80.0	1.4
Rye flour	84.0	84.0	84.0	5.6	5.6	0.0	5.5	71.4	4.1	75.5	89.1	0.6
Wheat flour	94.0	...	92.4	1.2	...	2.0	...	83.5	1.0	84.5	84.8	0.7
Rye Krysp	80.3	...	74.2	8.9	...	8.7	...	65.9	6.3	72.2	88.8	0.7
Wheat starch	111.1	...	...	0.0	...	...	...	100.0	0.0	100.0	...	...
Cornstarch	110.2	...	...	0.0	...	...	...	99.2	0.0	99.2	...	...

centage xylose was recorded as 3.2 (average of the four) in column 5 of Table I.

All 16 samples were extracted in triplicate. Repeated experiments on five of these samples, in which standards were run at the same time, confirmed the consistency of the method as shown in the fifth and sixth columns of Table I. Some experiments were run to find the effect of longer extraction time; no effect was observed for extractions which were continued for as long as 50 minutes. The percentage of pentose (as percentage of monosaccharide extracted) varied from 0.0 for wheat and corn starches, to 13.0 for oats (fifth and sixth columns of Table I).

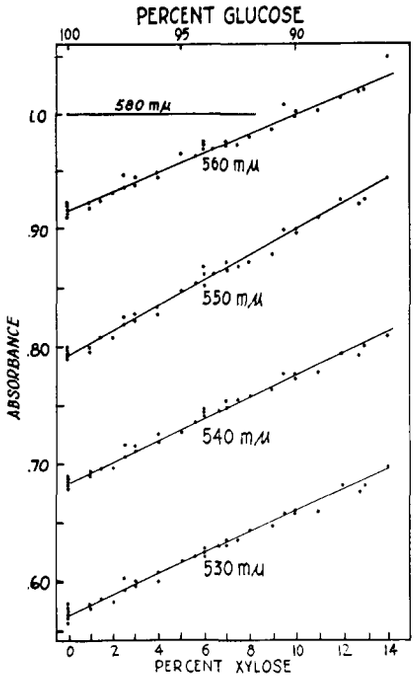

Figure 2. Effect of 0 to 14% xylose on sulfonated 1-naphthol-glucose reaction

Anthrone Reaction with Sulfonated 1-Naphthol Reaction. As the percentage of xylose was increased in the glucose-xylose mixtures, there was a decrease in the final color produced by the anthrone reaction, which resulted in a corresponding decrease in the absorbances at 620 and 630 mμ. Therefore, when a pentose is present, lower values are obtained (glucose standard) with the anthrone reaction. These facts offer a method of checking the results obtained as described in the preceding paragraphs. The percentage of sugar was estimated by both methods using glucose as the standard. The percentage difference was then multiplied by the factor 1.15 to arrive at the approximate xylose content. This factor, 1.15, was used because the pentose is responsible for some of the color (*7*). For example, with the sulfonated 1-naphthol reaction, the extract from 0.046 mg. of rye (dry weight) yielded products with an absorbance of 0.644 at 570 mμ and for 0.040 mg. of glucose, the absorbance was 0.700. This calculates as $\frac{0.644}{0.700} \times \frac{0.040}{0.046} \times 100 = 80.0\%$ as glucose in the rye. A similar calculation for the results from the anthrone reaction (at 620 mμ) indicates 74.5% as glucose in the rye. This latter value is $\frac{80.0 - 74.5}{80.0} \times 100 = 6.9\%$ lower than for the sulfonated 1-naphthol reaction. When the 6.9 is multiplied by 1.15, the percentage of pentose (percentage of extracted carbohydrate) is calculated to be 7.9. This value represents the percentage of the extracted carbohydrate which is pentose (calculated as xylose). The factor, 1.15, is an average value arrived at after making tests on a large number of standards containing 0 to 14% xylose in xylose-glucose mixtures.

In most cases, the percentages of pentose were nearly the same by both methods (see fifth to seventh columns of Table I). In cases where the two methods did not agree, the results from repeated experiments indicated that the greatest errors probably occurred more frequently because of inconsistencies of the anthrone method rather than the sulfonated 1-naphthol, as illustrated in Table I. This was probably because better controlled conditions were used (*7*, *9*) for the sulfonated 1-naphthol reaction.

Control of Conditions The sulfonated 1-naphthol test can be carried out rapidly because the sulfuric acid can be added to the tubes at 1-minute intervals and the tubes can then be removed from the boiling water bath one at a time. By such an arrangement, it is possible to make a large number of tests in a short time. The 8-minute heating period seems to give very good results, but it is essential to use controlled conditions. On hot days, when the tubes were removed from the ice bath and allowed to stand at room temperature for 20 minutes or longer, the color developed slightly on warming, and this slight change of color had a great effect on the pentose determination (when used in conjunction with the anthrone reaction). Therefore, the test on the sample must be carried out under the same conditions as the standard, if consistent results are to be obtained for estimation of pentoses, especially when used in conjunction with the anthrone reaction. Checks can be made by running a standard at the time the test is made on the sample.

The results in Table I are in close agreement with some earlier preliminary experiments, in which the heating time for the reaction was only 5 minutes and the hot mixture was allowed to stand for an hour at room temperature (*2*). This earlier method was abandoned because of the variation of room temperature, which seemed to cause somewhat inconsistent results in the pentose estimation.

Shorter heating periods (5 to 7 minutes) give better results if the rate of cooling is slow and nearly constant. The slow cooling rate permits more stable color development. When the 8-minute heating period is used and the colored mixture is cooled in ice water, the readings must be made quickly.

More consistent results are obtained when the sulfuric acid is added rapidly. Higher concentrations of carbohydrate give more consistent results than very low concentrations. The carbohydrate content of the standard should be near that of the sample.

A recent report by Scott and Melvin (*9*) on the anthrone reaction indicates that this reaction also requires carefully controlled conditions for precise work.

Summary and Conclusions

A simple, rapid test for the determination of carbohydrate in the sulfuric acid extract of cereals can be applied on an aliquot of the extract which is obtained during the crude fiber determination. The sulfonated 1-naphthol reaction is used in this test for estimating carbohydrate and percentage of pentose in the acid extract. The difference in carbohydrate content determined by the sulfonated 1-naphthol reaction, and that determined by the anthrone reaction, can be used to confirm the pentose content. The pentoses are present principally in the form of xylans and the nitrogen-free extracts of cereals and cereal grains contain substances other than carbohydrates. Both the anthrone (*7*, *9*) and sulfonated 1-naphthol reactions require control of variables for the most consistent results.

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