Total Starch Measurement in Cereal Products: Interlaboratory Evaluation of a Rapid Enzymic Test Procedure

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ABSTRACT

The precision of an enzymatic procedure for analysis of total starch in cereal flours and products was determined in a comprehensive interlaboratory study involving 29 laboratories. Test samples represented a range of sample types, including modified and native starches, cereal flours and brans, processed cereal products, animal feeds, and plant material. Results were statistically analyzed according to AOAC guidelines. The procedure was shown to be highly repeatable (relative standard deviation 1.5–7.3%) and reproducible (relative standard deviation 4.1–11.3%). It is now available, in a slightly modified form, as an assay kit. The assay, therefore, provides a convenient alternative to existing procedures for quantitative measurement of starch in cereal products.

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The quantitative analysis of total starch in many purified starch preparations and cereal products poses few problems for existing enzymic assay procedures (Batey 1982, AACC 1983, Aman and Hesselman 1984, Blakeney and Matheson 1984, Karkalis 1985, Henry et al 1990, Englyst et al 1992). However, these methods underestimate the starch content of materials containing starch that is resistant to amylolytic hydrolysis, e.g., high-amylose starches, chemically modified starches, and many processed cereal products containing resistant starch. Various pretreatment steps have been proposed to improve recoveries in such samples (Karkalis 1985, Henry et al 1990). The accuracy of chemical approaches (0.5*M* sodium hydroxide) to solubilize the starch before enzymic hydrolysis has been questioned (Kennedy and Cabalda 1993).

Most other nonenzymic methods for starch analysis are based on acid hydrolysis or dispersion in hot calcium chloride, followed by the polarographic or colorimetric measurement of resultant sugars. These methods are suitable for many purified starch samples (Analytical Working Party of the Starch Experts Group of the European Starch Associations 1987, Mitchell 1990), but they are less suitable for cereal flours or for samples with relatively low starch contents.

Recently, we developed an enzymic procedure for the analysis of total starch for use on a wide range of modified starches and starch-containing materials (McCleary et al 1994). In the procedure, starch is dispersed in dimethyl sulfoxide (DMSO) and then quantitatively hydrolyzed to glucose by sequential treatment with thermostable α-amylase, pullulanase-β-amylase, and amyloglucosidase (glucoamylase). The resultant glucose is measured colorimetrically with a glucose oxidase-peroxidase (GOPOD) reagent. The method is simple, rapid, and utilizes highly purified enzymes to ensure specific hydrolysis and measurement of starch.

The aim of this study was to evaluate the reproducibility and repeatability of the method for a range of starches and cereal products in an extensive interlaboratory evaluation by 29 collaborators.

MATERIALS AND METHODS

Total Starch Assay Kits

Full instructions and the following reagents were provided in kit form to each collaborator. The kits were based on the method developed by McCleary et al (1994) and were provided by Megazyme (Aust) Pty Ltd., Warriewood, Australia.

1. Thermostable α-amylase (EC 3.2.1.1) (10 ml, 400U/ml in 50% glycerol) purified from Termamyl 120 L (Novo Nordisk,

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Bioindustrial Group, Novo Alle 2880 Bagsvaerd, Denmark) by ion exchange and hydrophobic chromatography. This was diluted 20-fold with 50 m*M* MOPS buffer (pH 7.0) before use.

2. Pullulanase (EC 3.2.1.41) (100 U/ml) affinity purified from *Bacillus acidopullulyticus* plus β-amylase (EC 3.2.1.2) purified from *B. cereus* (500U/ml) in 3.2*M* ammonium sulfate (10 ml). This was diluted 40-fold with 100 m*M* sodium acetate buffer (pH 4.5) before use.

3. Amyloglucosidase (EC 3.2.1.3) (2 ml, 200 U/ml in 50% ammonium sulfate) purified to homogeneity by ion exchange and gel-permeation chromatography from *Aspergillus niger*. This was diluted 10-fold with 100 m*M* sodium acetate buffer (pH 5.0) before use.

4. Glucose oxidase-peroxidase-4-aminoantipyrine reagent (GOPOD) supplied as a freeze-dried powder and sufficient to prepare 1 L of reagent. Final reagent concentrations are >12,000 U/L glucose oxidase, >650 U/L peroxidase, and 0.4 m*M* 4-aminoantipyrine.

5. GOPOD reagent buffer concentrate (50 ml, 1*M* potassium phosphate (pH 7.4) containing 3% *p*-hydroxybenzoic acid and 0.4% sodium azide) sufficient to prepare 1 L of GOPOD reagent buffer. The buffer concentrate is diluted to 1 L and mixed with Reagent 4 before use.

6. Glucose standard solution (10 ml, 100 μg/0.1 ml in 0.2% aqueous [w/v] benzoic acid).

7. High-amylose maize starch for use as a control.

Test samples were chosen to represent the range of sample types commonly assayed for total starch and were either purchased from retail outlets or were provided by Weston Cereal Laboratories, Enfield, NSW; Goodman Fielder Wattie, Tamworth, NSW, and the Bread Research Institute of Australia Inc., North Ryde, NSW. They comprised maize starch (covalently cross-linked, regular, and high-amylose), oat bran, wheat flour, cake mix, bread, chicken feed, spaghetti, and green peas.

Assay Procedure

The assay procedure used in this study is detailed in McCleary et al (1994), with some minor modifications. Briefly, aqueous ethanol (0.2 ml, 50%, v/v) was added to 100 ± 5 mg of sample in a glass test tube to aid wetting. DMSO (1 ml) was then added with vigorous mixing, and the tube was heated for 5 min in a boiling water bath. Diluted thermostable α-amylase (2 ml, 40 U) was added with stirring, and the tubes were heated in a boiling water bath for a further 2 min. Diluted pullulanase–β-amylase solution (4.0 ml, 12.5 U and 50 U, respectively) was added with stirring, and the tube was incubated at 50°C for 60 min. The tube contents were then quantitatively transferred to a 100-ml volumetric flask, and the volume was adjusted with distilled water. Aliquots (0.1 ml) were mixed and then incubated with diluted amyloglucosidase solution (0.1 ml, 2 U) for 10 min at 50°C. GOPOD reagent (3 ml) was then added to each tube. Contents were mixed in a test tube stirrer, and the incubation was continued for another 20 min. The absorbance at 510 nm was measured against a reagent blank.

Calculations of Total Starch

Total starch is measured as the glucose derived from hydrolyzed starch and is expressed as a percentage of total sample weight on an as is basis.

$$\text{Total starch} = \Delta E \times F \times 1000 \times 1/1000 \times 100/W \times 162/180 = (\Delta E \times F)/\text{W} \times 90$$

where ΔE is the absorbance after amyloglucosidase treatment, read against the sample blank; F is a factor for the conversion of absorbance values to micrograms of glucose (100 μg of glucose/absorbance for 100 μg of glucose); 1000 is a volume correction factor (0.1 ml of 100 ml was analyzed); 1/1000 is a conversion from micrograms to milligrams; W is the sample weight; $100/W$ is a factor to express total starch as a percentage of sample weight; and 162/180 is a factor to convert free glucose to anhydroglucose, as occurs in starch. Starch content on an oven-dry basis was calculated using the moisture content of the sample reported by each collaborator.

Design of the Collaborative Study

Twenty homogenous test samples were provided as 10 blind duplicates to 29 collaborators, who were asked to become familiar with the method by repeated analysis of the reference sample supplied. Collaborators assayed each test sample in duplicate on an air-dry basis and provided the individual analyses, together with the determined sample moisture content. All results were adjusted for moisture content before statistical analysis. The test samples were chosen to represent a range of sample types and included unmodified and modified starches, cereal flours and brans, processed cereal products, and plant material.

The results were analyzed according to AOAC guidelines (AOAC 1990) using the Cochrans extreme variance test for repeatability and Grubbs test for reproducibility (both $P < 0.01$). These tests indicate test results that show a significantly greater variability among replicate (within-laboratory) analyses (Cochran), or the mean of replicate (between-laboratory) analyses (Grubbs), than the remaining test results. Outliers identified by these tests were omitted from the analysis of variance. Within (s_r) and between (s_R) laboratory standard deviations were determined from the analyses of variance of duplicate results for each sample. The repeatability value (r) represents the 95% confidence interval for repeat analyses under identical conditions in the same laboratory (2.8 s_r) and the reproducibility value (R) represents the 95% confidence interval for analyses on identical materials in separate laboratories (2.8 s_R). We also calculated relative standard deviations (RSD_r and RSD_R) from s_r and s_R as percentages of the mean values.

RESULTS AND DISCUSSION

Tables I and II show the total starch values determined by each collaborator as a percentage on a dry weight basis, as well as the mean, repeatability (r), repeatability relative standard deviation (RSD_r), reproducibility (R), and reproducibility relative standard deviation (RSD_R) for each test sample. More than four Cochran (repeatability) or Grubbs (reproducibility) test outliers were identified initially for collaborators 7 and 23. All results for these two collaborators were omitted from further calculations. In subsequent calculations, the Cochran test identified as outliers the results for the following samples: covalently cross-linked starch (collaborator 20), white bread (collaborators 12 and 20), high-amylose maize starch (collaborator 16), and white wheat flour (collaborator 16). The Grubbs test identified as outliers the results of collaborators 9, 14, and 28 for regular maize starch.

Analysis of the results yielded RSD_r values of between 1.5 and 7.3% and RSD_R values of between 4.1 and 11.3%. The nominal percent values given in Tables I and II represent the means of repeated assays of the samples on different occasions ($n = 4$) by the NSW Agriculture laboratories and are not included in the calculations.

RSD_R values were also predicted by the Horwitz equation (Horwitz et al 1990).

$$RSD_R \text{ (predicted)} = 2^{(1 - 0.5 \log C)}$$

where C is the starch concentration as a decimal fraction (1% = 0.01). The HORRAT ratios (Peeler et al 1989), determined as:

$$RSD_R \text{ (determined)}/RSD_R \text{ (predicted)}$$

yielded values ranging from 1.9 to 5.4, with a median of 2.5. This gives an index of acceptability of method performance. Horwitz regarded a HORRAT ratio of 2 or less to be satisfactory for analytical test procedures. However, this is rarely achieved in practice for procedures for the major components of food products (Horwitz et al 1990).

Few direct comparative precision data are available for starch analytical methods that have been subjected to an interlaboratory evaluation. The reproducibility is similar to that reported for an alkaline dispersion-enzymic procedure on purified starches (Analytical Working Party of the Starch Experts Group of the European Starch Associations 1987). Also, the RSD values and the HORRAT ratios determined in this study are equal to or superior to those for collaborative studies on methods of fiber and carbohydrate analysis in cereal products (Horwitz et al 1990). This is despite the use of a small test sample size (100 mg) and unfamiliarity of many of the collaborators with the test procedure which, in some cases, led to deviations from the assay format. The relatively high variability for analysis of the spaghetti sample was surprising, given the few problems found during development of the assay.

Several collaborators commented that the method was reliable and convenient to use, although one found the dilution step before amyloglucosidase treatment inconvenient for large sample numbers. This step could be performed by serial dilutions. One area of concern was the efficiency of mixing during α-amylase treatment, which requires a closely timed sequence for enzyme addition. Some samples tended to form gelatinous lumps if the procedure was not strictly followed. These comments were considered and, in general, implemented for the development of the final assay format (McCleary et al 1994).

CONCLUSIONS

The precision data for the total starch assay evaluated in this study demonstrate that the method provides a reliable and quantitative measure of starch in native and modified starch samples and processed cereal products. On the basis of these results, this method has been accepted as a standard procedure by the Cereal Chemistry Division of the Royal Australian Chemical Institute and has received first approval status by the American Association of Cereal Chemists. The availability of an assay kit that supplies standardized enzymes and reagents for the assay procedure offers a convenient and rapid alternative to existing assay procedures.

TABLE I
Collaborative Results[a] for Total Starch Determination (% dry weight) in Cereal Starch, Bran, and Flours

Lab No.	Covalently Cross-linked Maize Starch		Oat Bran		Regular Maize Starch		High-Amylose Maize Starch		White Wheat Flour	
1	97.2	96.0	50.8	50.2	101.9	102.7	100.3	102.8	81.7	83.1
2	95.2	90.1	44.4	45.4	94.7	95.7	98.3	97.2	78.6	79.4
3	96.1	98.1	49.1	47.0	94.9	95.9	97.2	94.1	84.9	81.7
4	91.3	93.7	47.7	48.8	100.5	100.6	94.3	96.6	81.5	87.5
5	93.4	102.4	43.7	54.3	97.9	105.0	108.6	107.2	88.6	89.2
6	97.3	93.2	38.2	37.9	99.1	95.8	95.7	96.8	77.8	80.1
7[b]	97.3	95.4	32.4	46.7	47.5[G]	60.5	98.4	97.7	46.6[G]	57.2
8	80.0	87.6	43.6	45.4	94.3	90.0	96.3	96.6	79.6	77.4
9	88.1	86.0	41.0	43.3	75.3[G]	82.7	86.6	87.5	71.9	73.9
10	96.1	93.4	47.8	47.6	98.1	98.7	98.9	99.3	70.3	77.9
11	93.3	90.1	45.9	51.5	97.3	95.5	97.6	95.6	79.2	78.4
12	91.7	95.5	38.8	46.9	101.0	96.7	95.8	95.3	80.6	80.4
13	97.4	94.3	50.8	50.1	102.9	107.1	107.5	103.9	82.8	89.9
14	88.3	83.0	50.8	36.4	90.3[G]	78.7	98.4	93.6	72.0	69.2
15	93.6	96.2	46.0	46.9	98.7	99.8	99.5	102.2	79.4	80.5
16	91.8	97.6	47.9	44.4	99.7	99.4	98.9[C]	86.6	91.8[C]	75.5
17	88.0	90.1	40.4	44.8	98.7	96.6	100.2	98.2	80.6	80.7
18	93.9	93.1	46.9	46.0	99.7	99.0	99.9	98.5	81.9	83.6
19	96.7	96.4	47.1	52.4	102.1	101.5	100.0	99.7	86.6	83.1
20	103.2[C]	50.9	47.9	53.3	99.0	105.0	109.4	112.5	...	86.2
21	93.1	88.2	46.3	47.1	97.2	97.3	96.5	94.9	78.3	78.1
22	95.5	95.1	43.8	45.4	97.2	98.4	97.4	96.7	79.4	80.2
23[b]	65.5[G]	74.3	37.7	37.3	68.4[C]	88.1	71.4[G]	59.6	58.9[G]	50.1
24	98.2	97.5	42.0	35.6	85.6	96.7	99.0	97.4	78.0	79.8
25	94.5	97.7	45.2	46.5	98.6	102.7	99.1	99.2	80.1	81.9
26	89.0	88.9	40.3	37.4	91.9	91.6	96.4	98.0	76.2	79.6
27	85.2	86.7	45.1	42.9	96.5	94.6	97.9	93.7	75.7	76.0
28	86.7	81.4	42.3	45.6	61.6[G]	87.5	92.0	93.2	79.5	77.7
29	95.1	93.3	49.0	48.8	100.8	108.5	97.6	96.7	81.4	83.3
Number of labs:	26		27		24		26		25	
Outliers	1		0		3		1		1	
Average %	92.5		45.6		98.4		98.2		80.0	
Nominal %	91.9		46.0		98.1		97.2		80.8	
r	7.6		9.3		7.8		4.2		5.9	
RSD_r	2.9		7.3		2.8		1.5		2.6	
R	13.2		12.0		11.7		13.4		12.2	
RSD_R	5.1		9.4		4.3		4.9		5.4	

[a] C = Cochran (repeatability) outlier ($P < 0.01$)
G = Grubbs (reproducibility) outlier ($P < 0.01$)
Labs = Number of laboratories included in calculations
Outliers = number of outlier laboratories, not included in calculations
Average % = arithmetic average result between laboratories
Nominal % = average of repeated determinations ($n = 4$) by the NSW Agriculture laboratories
r = repeatability (95% confidence interval for two single repeated tests)
RSD_r = relative standard deviation of repeatability within a laboratory
R = reproducibility (95% confidence interval for two single tests in different labs)
RSD_R = relative standard deviation of reproducibility between laboratories
[b] Results from collaborators 7 and 23 were omitted from calculations, having four or more outliers out of ten samples.

TABLE II
Collaborative Results[a] for Total Starch Determination (% dry weight) in Processed Cereal Products and Plant Material

Lab No.	Buttercake Cake Mix		White Bread		Chicken Feed Pellets		Spaghetti		Green Peas	
1	38.9	37.8	72.9	72.7	53.5	53.4	82.3	80.2	46.8	46.7
2	37.7	37.4	69.9	68.8	45.6	51.1	79.7	78.0	41.1	39.3
3	33.4	36.3	72.4	71.6	52.5	52.3	78.7	79.3	46.8	45.0
4	37.5	37.8	71.4	70.9	51.0	50.1	80.7	79.4	44.8	43.6
5	35.3	35.5	67.0	70.1	46.9	53.9	76.6	86.4	42.6	49.1
6	37.7	36.6	70.6	66.7	50.3	50.5	43.7[C]	73.1	44.6	45.2
7[b]	36.3[G]	18.7	79.5	75.5	36.6	42.9	59.8	80.8	37.4[G]	34.2
8	36.3	34.2	68.7	68.3	37.9	35.5	79.3	72.5	41.4	41.9
9	34.1	33.2	63.6	63.2	45.5	45.0	71.8	56.3	43.5	39.9
10	37.8	37.8	72.9	73.4	45.1	48.3	77.8	74.5	44.7	44.1
11	33.8	35.9	68.5	70.3	47.0	47.6	77.7	79.1	44.9	44.3
12	34.7	34.6	62.1[C]	69.9	49.9	50.5	77.0	77.0	44.0	42.4
13	38.1	44.8	72.0	74.3	45.8	44.2	58.2	64.9	48.5	51.6
14	40.6	38.6	74.8	73.4	53.9	45.6	78.6	65.7	42.8	40.8
15	36.3	39.7	71.4	71.2	53.3	53.6	79.8	78.2	45.1	45.8
16	37.3	38.1	68.5	68.4	41.3	41.4	79.8	74.5	43.7	45.2
17	37.4	37.6	69.1	68.7	45.4	44.0	77.2	78.5	43.9	43.9
18	39.1	39.6	66.8	68.2	50.0	52.5	79.9	78.4	45.5	45.8
19	37.7	43.0	72.2	71.3	50.9	52.2	82.8	86.7	49.6	46.0
20	35.9	39.7	73.2[C]	58.5	53.9	55.6	82.9	84.2	47.3	44.2
21	35.7	35.4	68.2	69.2	50.1	47.4	79.0	77.4	44.0	44.6
22	36.3	35.7	70.5	68.9	38.2	49.2	66.2	76.2	43.3	43.1
23[b]	30.0[G]	30.1	50.5[G]	52.9	35.0	30.2	55.1	50.1	33.5[G]	34.6
24	35.8	38.3	76.7	74.5	41.8	34.0	61.4	43.7	39.6	43.7
25	35.8	38.3	66.7	66.2	45.7	47.6	77.6	79.7	45.7	44.9
26	32.9	36.1	67.3	69.6	38.4	38.4	61.0	55.1	42.9	43.0
27	32.7	28.5	66.0	66.0	46.5	42.6	75.7	72.6	41.0	39.7
28	32.6	37.3	66.6	67.4	48.1	49.2	75.7	73.4	41.8	41.4
29	39.2	37.2	69.8	70.4	51.0	51.8	80.0	77.1	43.0	46.1
Number of labs:	27		25		27		26		27	
Outliers	0		2		0		1		0	
Average %	36.8		69.8		47.5		75.1		44.2	
Nominal %	38.6		67.2		52.1		76.5		44.2	
r	5.3		3.0		7.5		13.1		4.4	
RSD_r	5.1		1.5		5.7		6.2		3.6	
R	7.4		8.1		14.6		23.7		7.1	
RSD_R	7.2		4.1		10.9		11.3		5.7	

[a]C = Cochran (repeatability) outlier ($P < 0.01$)
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Labs = Number of laboratories included in calculations
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R = reproducibility (95% confidence interval for two single tests in different labs)
RSD_R = relative standard deviation of reproducibility between laboratories
[b]Results from collaborators 7 and 23 were omitted from calculations, having four or more outliers out of ten samples.

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