

Variability in a Starch Isolation Method and Automated Digital Image Analysis System Used for the Study of Starch Size Distributions in Wheat Flour

Donald B. Bechtel^{1,2} and Jeff Wilson¹

ABSTRACT

Cereal Chem. 77(3):401–405

A starch isolation method and digital image analysis system were developed to accurately measure size distributions of the starch populations in wheat. The image analysis system was coupled directly to a light microscope equipped with computer controlled step stage and automatic focus. Automation of data acquisition and processing eliminated some of the labor intensive steps previously required for analyzing starch granule size distributions. This system was used to standardize starch isolation methods

and compare variation and precision of the system. Operational variations were determined and statistically assessed. The number of fields of view required for low standard errors and acceptable speed of analysis was determined to be fifty. A major advantage of the system has been the increased resolution. The use of higher magnifications and stage automation allowed the analysis of starch granules as small as 0.84 μm in diameter while analyzing thousands of starch granules per sample.

The most abundant component in wheat grain is starch, comprising ≈ 54 –62% of wheat grain (Pomeranz and McMasters 1968). Wheat starch is unique as it cannot be replaced by corn, rice, or oat starches or by noncereal starches to yield a satisfactory baked product (Hoseney et al 1971). Various reports have linked starch granule size to different rheological properties (Kulp 1973, Casey et al 1997, Rasper and deMan 1980), baking characteristics (D'Appolonia and Gilles 1971), and compositional differences (Meredith 1981). It is necessary to have an accurate method that measures the entire range of sizes of granules in a preparation to routinely quantify starch granule size distributions.

A number of methods has been used to measure size distributions of wheat starch including microsieving, electrical-sensing zone techniques, light scattering, and quantitative image analyses (Brookhurst and Evers 1977; Evers and Lindley 1977; Baruch et al 1979, 1983; Karlsson et al 1983; Soulaka and Morrison 1985; Morrison and Scott 1986; Bechtel et al 1990; Raeker et al 1998; Peng et al 1999; Stoddard 1999). Each method has advantages and disadvantages (Langton and Hermansson, 1993), but only digital image analysis coupled to light microscopy offers the ability to have physical parameters recorded for each individual particle and be able to distinguish among individual granules, agglomerated granules, and nonstarch particles.

The present study reports on methodology for the development of an automated image analysis system for measuring starch granule size distributions. One of the most important aspects in developing an image analysis system is determining the errors and precision associated with that system. We report here on the development of a fast and consistent starch isolation method, an automated digital image analysis system with minimal operator input, and the ability to measure starch granules $< 1 \mu\text{m}$ in diameter. We have determined the amount of variation and precision within the system and have determined the minimum number of analyses needed for routine measurements.

MATERIALS AND METHODS

Starch Isolation From Flour

Starch was isolated from flour using a modified protein digestion procedure (Brookhurst and Evers 1977, Morrison and Scott 1986) from a wheat flour (12.1% protein; 13.0% moisture content). Flour (0.3 g) was placed in 50-mL plastic centrifuge tubes with 5.0 mL of water and 2 mL of 0.8% pepsin A (P7012, Sigma, St. Louis, MO) in 0.04N HCl and incubated for 60 min at 37°C. After protease treatment, 1.0 mL of 0.08% Hemicellulase 90 (90,000 U/g activity, a gift from Amano Enzyme U.S.A., Lombard, IL) in 0.1M sodium acetate buffer (pH 4.5) was added to the mixture and incubated for 3 hr at 45°C. A detergent mix (1 mL) (5% SDS, 5% Triton X-100, 5% Tween 40, and 5% Triton X-15) was added after incubation, and the suspension was vortex-mixed for 30 sec. The enzyme-treated starch was centrifuged at 2,500 rpm for 5 min in a Sorval SS-34 rotor and SS-3 centrifuge. The starch was washed twice with water; the supernatant was discarded and the starch pellet was resuspended in 5 mL of water followed by vortex mixing for 30 sec and centrifuging at 2,500 rpm for 5 min. A final water wash was conducted in a microcentrifuge tube with 1.0 mL of water and centrifuged for 1 min. The isolation method was conducted on the same flour by two researchers to determine reproducibility, and the starch was lyophilized and weighed to determine extraction rates. Starch used for image analysis was not normally dried, as drying tended to increase clumping of the starch granules on slide preparations. Instead, after the final wash, the supernatant was discarded and 1.0 mL of 1% sodium azide was added and mixed followed by a brief centrifugation. The sodium azide supernatant was decanted and the isolated starch was stored at 4°C. Sodium azide was used to prevent microbial contamination and had the added benefit of decreasing clumping of the starch during staining and microscope slide preparation for image analysis.

Starch Staining and Image Analysis

Starch was used either stained or unstained for image analysis. Dark-field light microscopy was utilized if unstained starch was used, while bright-field illumination was used with stained starch samples. Both stained and unstained starch gave similar results, but starch that was stained seemed less prone to edge effects caused by illumination in the microscope. Isolated starch was mixed with 1.0 mL of water to make a slurry before using. Staining of isolated starch was accomplished by placing two drops of a water slurry of starch in a microcentrifuge tube and two drops of 1% periodic acid. The mixture was vortex-mixed briefly and 15 min later, 2 drops of 3% iodine and potassium iodide solution was added and mixed. The volume was then adjusted to 1 mL with distilled water, and the starch was ready to be mounted onto glass slides. Just before making

¹ USDA, ARS, Grain Marketing and Production Research Center, Grain Marketing Research Laboratory, 1515 College Ave. Manhattan, KS 66502. Names are necessary to report factually on available data; however, the USDA neither guarantees nor warrants the standard of the product, and the use of the name by the USDA implies no approval of the product to the exclusion of others that may also be suitable.

² Corresponding author. E-mail: don@usgmrl.ksu.edu

slides, the sample was placed into suspension by a vortex mixer, a drop was placed on a 1- × 25- × 75-mm glass slide, and a 22- × 50-mm cover glass was used to spread and cover the starch suspension. The cover glass was then ringed with Cytoseal 60 (Stephens Scientific, Riverdale, NJ) to prevent drying of the slide during image analysis. Great care needed to be taken during the mounting process to assure that the starch was evenly distributed and lacked air bubbles.

The slides were analyzed under dark-field (unstained starch) or bright-field (stained starch) illumination with a Reichert Polyvar 2 microscope equipped with a MAC 2000 (Ludl Electronics Products, Hawthorne, NY) automated stage attached to the microscope, allowing precise image acquisition and automatic focusing under computer control. Images were captured with a Javelin Chromachip V high-resolution CCD color camera coupled to a Javelin CVM nine color monitor and a Princeton Gamma-Tech Imagist II Imaging System (Vers. 7.1, Princeton Gamma-Tech, Princeton, NJ). The imaging system was operated on a SUN SPARC station, with a 32-bit processor, 32-Mb of main memory with both image acquisition and data analysis being done concomitantly. A 5-GB external hard disk was used to store images and data. The number of samples analyzed in a day has recently been increased threefold by acquiring images during the workday and analyzing the data at night rather than doing both at the time of sample analysis.

Starch granules were analyzed with a 25× objective that allowed for analysis of particles as small as 0.84 μm in diameter. The stage automation software was set to collect 20 random images from the sample area on the microscope slide. The stage moved to a random point and the sample was automatically focused. A gray scale image was captured and stored. The digital image was used to develop a color table to differentiate between the starch granules and back-

ground. The high contrast of starch against the background for either bright- or dark-field situation resulted in the use of simple color tables. All pixels with intensity above a set intensity value were displayed in one color while the background was black. A binary starch intensity level was chosen to yield starch granule measured diameters similar to those of the gray scale image. The color table was modified until both images resulted in measurement differences of only one or two pixels for the large A-type granules. The same color table was used for all dark-field illuminated samples, with a separate one developed for stained starch. All microscope viewing parameters were kept constant for each microscope viewing setup.

The binary images were processed using the PGT particle segmentation program to digitally separate touching particles. Erosion and dilation programs were evaluated and found to be inadequate and too time-consuming. Starch granules touching the edge of the field of view were eliminated from analysis and not counted. The stored binary data was used to calculate a number of parameters

TABLE I
Starch Isolation

Sample	% Recovery 1	% Recovery 2
1	82.3	84.1
2	83.5	84.6
3	82.9	83.7
4	83.8	84.7
5	83.6	80.2 ^a
6	83.3	83.0
7	82.1 ^a	84.4
8	83.2	85.4
9	81.9 ^a	84.1
10	82.6	85.4

^a Small amount of starch lost during lyophilization.

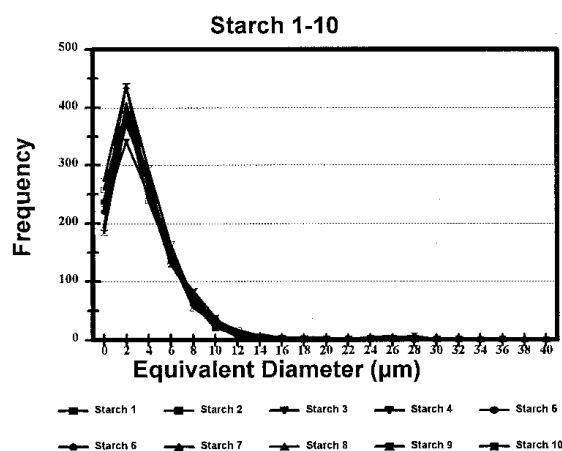


Fig. 1. Size-distribution plot of 10 sets of 20 random fields of view from a single slide and single starch isolation plotted as frequency vs. equivalent diameter.

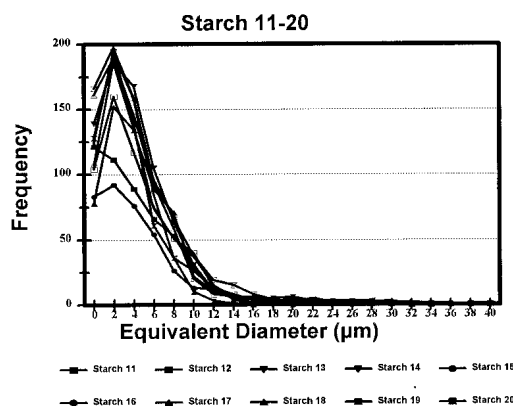


Fig. 3. Size-distribution data from 20 random fields of view from 10 different slides of a single starch isolation plotted as frequency vs. equivalent diameter.

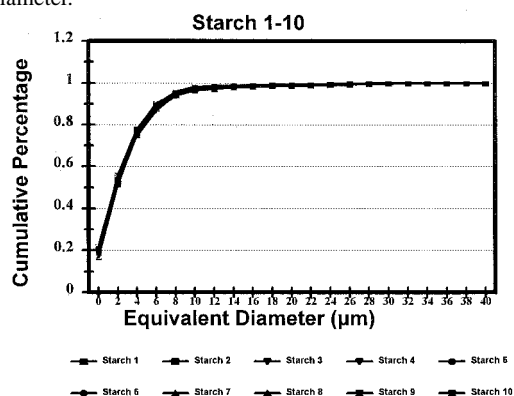


Fig. 2. Plot of cumulative percentage vs. equivalent diameter to normalize data for variations in number of granules measured for data in Fig. 1.

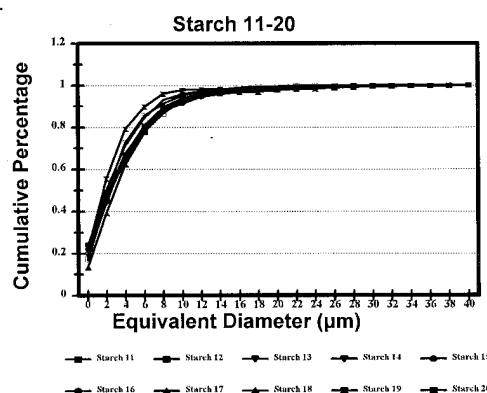


Fig. 4. Plot of cumulative percentage vs. equivalent diameter to normalize data for variations in number of granules measured for data in Fig. 3.

stored binary data was used to calculate a number of parameters including circularity shape factor, equivalent diameter, equivalent area, roughness, and aspect ratio (see Bechtel et al 1990 for definition of terms

RESULTS AND DISCUSSION

One of the most important aspects of a study on starch granule size distribution is to isolate all of the starch from the sample, particularly the small granules (South and Morrison 1990). Assuming that starchy endosperm comprised 81.4–84.1% of the whole wheat grain (Pomeranz 1988) and starch made up 63–72% of the caryopsis (Lineback and Rasper 1988), starch content of wheat flour should be 77–85% by weight. Starch isolated from flour using our method of enzymes and detergents gave consistent recoveries of 80.2–85.4% (Table I). Similar starch recoveries were obtained using a maceration and cesium chloride centrifugation method (Morrison and Gadan 1987, South and Morrison 1990). Yields of those magnitudes indicate that nearly all of the starch has been isolated from the flour, including the small granules usually retained in the fraction called tailings.

A major goal of using image analysis technology was to determine the reproducibility and amount of variation of the analyses both within a sample and among samples. To address those issues three sets of data were gathered and analyzed: 1) 10 sets of 20 random fields of view from a single slide and single starch isolation; 2) 20 random fields of view from 10 different slides of a single starch isolation; 3) 20 random fields of view from 10 slides of 10 dif-

ferent starch isolations. The amount of variation on a single slide was determined using a frequency graph plotting number of starch granules measured versus equivalent diameter for the 10 sets of 20 fields of view from a single starch isolation showed nearly identical curves for each set of data (Fig. 1). Variation in the height of the peak centered at $\approx 2 \mu\text{m}$ was related to the number of granules measured. Data normalized by using cumulative percent eliminated the effect of variation in the number of analyzed starch granules and revealed little variation among the 10 sets of data (Fig. 2). Statistical analysis of the data obtained from the 10 sets revealed low standard errors for all parameters (Table II). The data indicated that the methods used to prepare the slides produced uniform spreading of starch granules on the slide and that randomly selected fields of view gave reproducible size distributions with low standard errors.

The amount of variability and reproducibility among different slides was checked by using slides made from a single starch isolation with varying amounts of starch on each slide. Ten sets of data from 10 different slides of one starch isolation each with 20 random fields of view showed greater variation than sets taken from only one slide (Fig. 3, Table III). Normalizing that data by using cumulative percent eliminated the effect of variation in the number of starch granules analyzed and showed most of the variation occurred at 2–12 μm starch diameter (Fig. 4). Statistical analysis showed that the standard error increased the greatest for area, perimeter, and circularity shape factor, and increased the least for aspect ratio and equivalent diameter. While the number of starch granules varied twofold (377–774, Table III) on the 10 slides measured, the reproducibility was still good.

TABLE II
Values for 20 Fields of View for 10 Random Sets from a Single Slide and from a Single Starch Isolation

Random Set	Granules	Average Values				
		Area	Perimeter	Aspect Ratio	Equivalent Diameter	Circularity Shape Factor
1	1,094	25.1	15.6	1.31	4.59	1.21
2	1,191	30.4	16.6	1.36	4.66	1.27
3	1,138	26.4	16.0	1.35	4.60	1.23
4	1,204	31.0	17.0	1.41	4.75	1.31
5	1,170	27.1	16.1	1.32	4.62	1.22
6	1,154	27.7	16.3	1.33	4.70	1.23
7	1,298	26.8	15.9	1.36	4.51	1.23
8	1,088	28.5	16.5	1.36	4.70	1.23
9	1,185	26.4	16.2	1.41	4.56	1.28
10	1,143	28.2	15.8	1.36	4.58	1.25
Total granules	11,665					
Mean	1,166.5	27.8	16.2	1.36	4.63	1.25
Standard error	19.0	0.6	0.1	0.01	0.02	0.01
Median	1,162.0	27.4	16.1	1.36	4.61	1.23
Standard deviation	60.2	1.8	0.4	0.03	0.07	0.03
Confidence level (0.95)	37.3	1.1	0.3	0.02	0.05	0.02

TABLE III
Values for 20 Fields of View for 10 Random Sets from 10 Slides and from a Single Starch Isolation

Random Set	Granules	Average Values				
		Area	Perimeter	Aspect Ratio	Equivalent Diameter	Circularity Shape Factor
11	774	34.3	20.4	1.47	5.27	1.63
12	583	28.1	15.4	1.40	4.48	1.21
13	698	31.8	17.2	1.44	4.88	1.28
14	585	49.1	20.6	1.40	6.02	1.19
15	638	46.1	20.1	1.42	5.75	1.26
16	377	42.4	20.2	1.53	5.51	1.35
17	655	32.9	17.2	1.36	5.03	1.21
18	739	40.6	19.2	1.48	5.51	1.31
19	586	41.2	19.8	1.40	5.66	1.27
20	499	45.6	19.4	1.40	5.60	1.24
Total granules	6,134					
Mean	613.4	39.2	19.0	1.43	5.37	1.30
Standard error	36.9	2.2	0.6	0.02	0.15	0.04
Median	612.0	40.9	19.6	1.41	5.51	1.27
Standard deviation	116.7	7.0	1.7	0.05	0.46	0.13
Confidence level (0.95)	72.3	4.4	1.1	0.03	0.29	0.08

TABLE IV
Values for 20 Fields of View for 10 Random Sets from 10 Slides and from 10 Separate Starch Isolations

Random Set	Granules	Average Values				
		Area	Perimeter	Aspect Ratio	Equivalent Diameter	Circularity Shape Factor
21	774	34.7	19.1	1.41	5.45	1.32
22	1,347	33.0	18.4	1.42	5.20	1.34
23	826	35.9	18.4	1.41	5.24	1.35
24	433	35.3	18.7	1.34	5.46	1.27
25	596	35.2	18.3	1.33	5.35	1.25
26	294	55.7	22.9	1.54	6.44	1.40
27	486	48.2	21.3	1.40	6.00	1.35
28	349	39.5	20.2	1.46	5.62	1.42
29	260	49.1	23.8	1.46	6.43	1.57
30	557	49.6	22.5	1.42	6.27	1.40
Total granules	5,922					
Mean	592.2	41.6	20.4	1.42	5.75	1.37
Standard error	102.9	2.6	0.7	0.02	0.16	0.03
Median	521.5	37.7	19.6	1.41	5.54	1.35
Standard deviation	325.5	8.2	2.1	0.06	0.49	0.09
Confidence level (0.95)	201.8	5.1	1.3	0.04	0.31	0.06

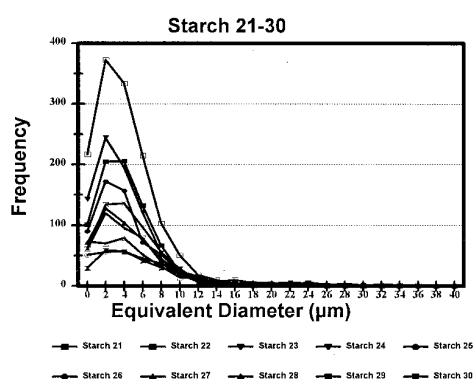


Fig. 5. Size-distribution data from 20 random fields of view from 10 slides of 10 different starch isolations plotted as frequency vs. equivalent diameter.

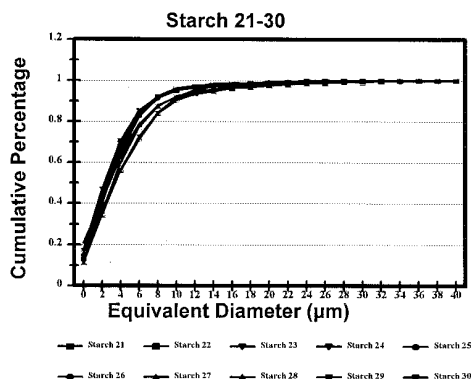


Fig. 6. Plot of cumulative percentage vs. equivalent diameter to normalize data for variations in number of granules measured for data in Fig. 5.

The amount of variation from starch isolation to starch isolation, as well as the influence of the number of starch granules analyzed was also investigated. Data from 20 fields of view of 10 slides from 10 different starch isolations showed the most variation (Fig. 5, Table IV). While the curves seemed substantially different, normalizing the data for variations in number of granules analyzed by plotting cumulative percent showed that data did not differ significantly from that of a single isolation (compare Figs. 4 and 6). Statistical analysis showed only slight increases in the standard error for most parameters and a minor decrease in that for circularity shape factor. Therefore, the data indicate that the shape of nonnormalized size-distribution curves cannot be used to compare starch from dif-

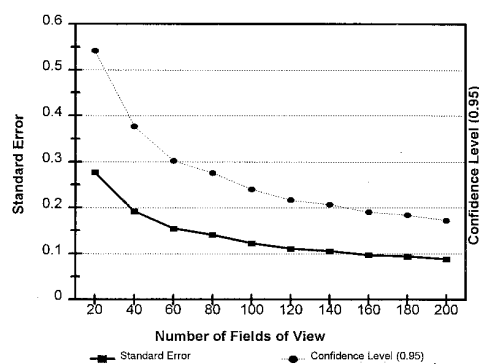


Fig. 7. Data in Fig. 6 reformatted and plotted as number of fields of views vs. standard error and confidence level.

ferent wheat samples. Normalization can be handled by either counting the same number of granules for each analysis or by a mathematical normalization as was done in this study. We chose to count the same number of fields of view and vary the number of granules counted on each slide to account for distribution variation of starch on slides and to determine what effect the number of starch granules measured had on the size distribution. The three sets of data (Figs. 1–6) clearly showed that, once it was normalized, reproducibility of both the starch isolation method and image analysis system was very good.

For this image analysis system to be used for routine assays, it is important to know how many fields of view are needed for reproducible results with the least amount of variation. Having the fewest number of observations possible was of the utmost importance because of time constraints required for the instrumentation to find each field of view, focus, acquire the image, and analyze the data. The more fields of view required meant fewer samples that could be analyzed. The number of fields of view needed was determined by using the data from the last set of 200 starch images.

That data represented the most variation using 10 slides from 10 separate starch isolations. The raw data was reformatted and was plotted as the number of fields of view analyzed versus the standard error and confidence level. Analysis showed that both standard error and confidence level decreased the most between 20 and 60 fields of view and then continued to decrease to a minimum at 200 fields of view (Fig. 7). From this data, the number of views needed to obtain the least variable and most reproducible data was 100–200 fields of view. The large number of fields of view needed for a low standard error was offset, however, by the amount of time required by the instrumentation to acquire and process the data (≈ 1.5 min/field of

view). Based on these two contradictory criteria, it was decided that 50 fields of view on two slides (total of 100 fields of view) was a compromise. We have recently increased the number of samples analyzed per day by acquiring images during the day and analyzing the data at night.

In summary, we have developed a reproducible method of starch isolation for use with an image analysis system for the routine assaying of starch size distributions in wheat. We have also identified the variation and precision within the image analysis system and can now apply these methods to analyze wheats differing in end-use properties.

LITERATURE CITED

- Baruch, D. W., Meredith, P., Jenkins, L. D., and Simmons, L. D. 1979. Starch granules of developing wheat kernels. *Cereal Chem.* 56:554-558.
- Baruch, D. W., Jenkins, L. D., Dengate, H. N., and Meredith, P. 1983. Nonlinear model of wheat starch granule distribution at several stages of development. *Cereal Chem.* 60:32-35.
- Bechtel, D. B., Zayas, I. Y., Kaleikau, L., and Pomeranz, Y. 1990. Size-distribution of wheat starch granules during endosperm development. *Cereal Chem.* 67:59-63.
- Brocklehurst, P. A., and Evers, A. D. 1977. The size distribution of starch granules in endosperm of different sized kernels of the wheat cultivar Maris Huntsman. *J. Sci. Food Agric.* 28:1084-1089.
- Casey, B. N., Warthesen, J. J., and Miller, L. C. 1997. Effect of different wheat starch granule characteristics on mixing properties in a dough system. *Cereal Foods World* 42:669.
- D'Appolonia, B. L., and Gilles, K. A. 1971. The effect of various starches in baking. *Cereal Chem.* 48:625-636.
- Evers, A. D., and Lindley, J. 1977. The particle-size distribution in wheat endosperm starch. *J. Sci. Food Agric.* 28:98-102.
- Hoseney, R. C., Finney, K. F., Pomeranz, Y., and Shogren, M. D. 1971. Functional (breadmaking) and biochemical properties of wheat flour components. VIII. Starch. *Cereal Chem.* 48:191-201.
- Karlsson, R., Olered, R., and Eliasson, A.-C. 1983. Changes in starch granule size distribution and starch gelatinization properties during development and maturation of wheat, barley and rye. *Starch* 35:335-340.
- Kulp, K. 1973. Characteristics of small-granule starch of flour and wheat. *Cereal Chem.* 50:666-679.
- Langton, M., and Hermansson, A. 1993. Image analysis determination of particle size distribution. *Food Hydrocoll.* 7:11-22.
- Lineback, D. R., and Rasper, V. F. 1988. Wheat carbohydrates. Pages 277-372 in: *Wheat Chemistry and Technology*, Vol. I. Y. Pomeranz, ed. Am. Assoc. Cereal Chem.: St. Paul, MN.
- Meredith, P. 1981. Large and small starch granules in wheat—Are they really different? *Starch/Staerke* 33:40-44.
- Morrison, W. R., and Scott, D. C. 1986. Measurement of the dimensions of wheat starch granule populations using a Coulter Counter with 100-channel analyzer. *J. Cereal Sci.* 4:13-21.
- Morrison, W. R., and Gadan, H. 1987. The amylose and lipid contents of starch granules in developing wheat endosperm. *J. Cereal Sci.* 5:263-275.
- Peng, M., Gao, M., Abdel-Aal, E.-S. M., Hucl, P., and Chibbar, R. N. 1999. Separation and characterization of A- and B-type starch granules in wheat endosperm. *Cereal Chem.* 76:375-379.
- Pomeranz, Y. 1988. Chemical composition of kernel structures. Pages 97-158 in: *Wheat Chemistry and Technology*, Vol. I. Y. Pomeranz, ed. Am. Assoc. Cereal Chem.: St. Paul, MN.
- Pomeranz, Y., and McMasters, M. M. 1968. Structure and composition of the wheat kernel. *Baker's Dig.* 42(4):24-29,32.
- Raeker, M. Ö., Gaines, C. S., Finney, P. L., and Donelson, T. 1998. Granule size distribution and chemical composition of starches from 12 soft wheat cultivars. *Cereal Chem.* 75:721-728.
- Rasper, V. F., and Deman, J. M. 1980. Effect of granule size of substituted starches on the rheological character of composite doughs. *Cereal Chem.* 57:331-340.
- Soulaka, A. B., and Morrison, W. R. 1985. The amylose and lipid contents, dimensions, and gelatinization characteristics of some wheat starches and their A- and B-granule fractions. *J. Sci. Food Agric.* 36:709-718.
- South, J. B., and Morrison, W. R. 1990. Isolation and analysis of starch from single kernels of wheat and barley. *J. Cereal Sci.* 12:43-51.
- Stoddard, F. L. 1999. Survey of starch particle-size distribution in wheat and related species. *Cereal Chem.* 76:145-149.

[Received October 22, 1999. Accepted January 20, 2000.]