MODIFIED WOHLGEMUTH METHODS FOR ALPHA-AMYLASE ACTIVITY OF WHEAT AND RYE[1]

SVEN HAGBERG[2]

ABSTRACT

Modified methods for determining alpha-amylase activity of wheat and rye grain or flour based on the Wohlgemuth principle are described. These can be carried out comparatively rapidly and give values in SKB units. The end-point is determined by using either a permanent color standard or by taking colorimetric readings at fixed time intervals.

Among methods for determining alpha-amylase activity based on the Wohlgemuth principle, the method of Sandstedt, Kneen, and Blish (16) as described in *Cereal Laboratory Methods* (1) has achieved widespread popularity. This method was originally intended for malt, but modifications for wheat and rye have been suggested by Kneen and Sandstedt (8), Kneen, Sandstedt, and Hollenbeck (9), Olson, Evans, and Dickson (13), Redfern (14), and Stone (17). In Germany and some other countries the methods of Ritter (15) and Lemmerzahl (11) are used. These modified Wohlgemuth methods are either time-consuming and difficult to carry out, or the relation between the values obtained and the well-known SKB units have not been determined.

Hanes and Cattle (4) were the first to show that the measurement of absorbancy could be used to follow quantitatively the alteration in the iodine coloring property of starch during its degradation by amylases. Bawden and Artis (2) have reported colorimetric methods for evaluating alpha-amylase activity of malt. Similar methods for wheat and rye have been used by Hoskam (5), Jongh (6), Knight (10), Olerod (12), and Hagberg (3).

The object of the present study was to develop rapid, accurate methods based on the Wohlgemuth principle for determination of alpha-amylase activity of wheat and rye and its expression in SKB units.

Materials

Two samples each of wheat, wheat flour, rye, and rye flour were used in this study. Each pair of samples was blended to give three additional samples with activities ranging between those of the unmixed samples (see Table I).

The wheat and rye samples were finely pulverized according to

[1] Manuscript received February 2, 1960.
[2] Late Head, Institution of Food Chemistry, Statens Hantverksinstitut, Stockholm 4, Sweden.

TABLE I

ANALYTICAL DATA FOR WHEAT, RYE, AND FLOUR SAMPLES

MATERIAL	SAMPLE No.	ASH	PROTEIN	ALPHA-AMYLASE ACTIVITY
		%	%	*SKB units*
Wheat flour	1	0.52	12.3	0.06
Wheat flour	5	0.66	9.6	6.00
Rye flour	6	0.77	8.7	0.13
Rye flour	10	0.68	7.0	8.10
Ground whole wheat	11	..	..	0.08
Ground whole wheat	15	..	..	1.53
Ground whole rye	16	..	..	0.05
Ground whole rye	20	..	..	4.34

Cereal Laboratory Methods (1). Ninety percent of the ground material passed through a No. 30 standard sieve.

I. A Modified AACC Method for Determination of Alpha-Amylase

This method is performed similarly to that described in *Cereal Laboratory Methods* (1) but using the glass disk No. 620-S5 in a Hellige Comparator No. 607 or comparable device as suggested by Redfern (14). The Comparator should be illuminated by an ordinary 100-watt frosted bulb mounted 6 in. from the rear opal glass of the Comparator, in such a manner that direct rays from the lamp do not shine in the operator's eyes. Laboratories which do not have the Hellige equipment may use a stable colored solution giving the same results as the Hellige disk. The solution suggested by Landis and modified by Olson, Evans, and Dickson (13) gives a more reddish color than the Hellige disk, when an ordinary 100-watt frosted bulb is used. However, the following solution gives practically the same color as disk No. 620-S5 when compared in the Hellige Comparator: 19.250 g. cobaltous chloride hexahydrate ($CoCl_2 \cdot 6H_2O$), 2.567 g. potassium dichromate, and 2.565 g. cupric sulfate pentahydrate per 100 ml. 0.01*N* hydrochloric acid solution.

Apparatus and Reagents. The apparatus and reagents are the same as those described in *Cereal Laboratory Methods* (1), with the following exceptions:

(1) Dilute iodine solution. Dissolve 30 g. potassium iodide in water, add 3 ml. stock iodine solution, and make up to 500 ml. This solution should be at, or close to, 30°C. when color comparisons are made. Make fresh solution daily.

(2) Calcium chloride solution. Use 0.2% solution for all extracts.

(3) Buffered beta-amylase limit dextrin solution. This may be the same as described in *Cereal Laboratory Methods* (1) or it may be a buffered solution containing an equivalent amount of beta-amylase limit dextrin (erythrodextrin) in the solid state prepared as described by Hoskam (5) and Jongh

(6).[3] One gram of "starch" as understood in the AACC method (1) is equivalent to about 0.4 g. of erythrodextrin on a dry-weight basis.

Preparation of Extract. Extract 5 g. of flour or finely pulverized grain with 100 ml. of 0.2% calcium chloride solution for 1 hour at 30°C., mixing by rotation every 15 minutes. Centrifuge at 2,500 r.p.m. (Corda centrifuge for 100-ml. tubes, mean radius 10 cm.) for 10 minutes and filter the supernatant liquor through No. 42 Whatman filter paper. Refilter the first part of the filtrate until a clear filtrate is obtained. Dilute the extract suitably with 0.2% calcium chloride solution. Bring this extract to 30°C. before making the activity determination.

Determination of Alpha-Amylase Activity. Transfer 30 ml. of the extract to a 150-ml. test tube (about 32 by 200 mm.) or 125-ml. Erlenmeyer flask, and place in the 30°C. bath. After attempering to 30°C. add 10 ml. of the buffered beta-amylase limit dextrin solution and mix well. The relation between the volumes of extract (diluted if needed) and the dextrin solution should always be 3 to 1.

A. Ordinary Procedure for End-Point Determination (Modified AACC Method IA).

A series of test tubes (13 by 100-mm.) containing 5 ml. dilute iodine solution are prepared and attempered at 30°C. in readiness for testing. At appropriate intervals, 4 ml. of the hydrolyzing mixture are added from a fast-flowing pipet to the 5 ml. of iodine solution. It is important in this and all other similar methods that the pipet is not polluted with iodine during draining. This can be avoided by draining the pipet on the upper part of the test tube in which the iodine solution previously has been carefully conveyed by aid of a pipet into the lower part of the test tube. After mixing, the hydrolysate-iodine solution is poured into the 13-mm.-square tube for color comparison in the Hellige Comparator. After the color comparison is made, the solution is poured out by giving the tube a quick shake. In this way very little liquid remains in the tube and it is ready for another test. When the end-point is near, readings are made every 0.5-minute if the hydrolyzing time is 10 to 30 minutes, and the dextrinizing time is interpolated as the 0.25-minute between two readings (14).

For convenience it is desirable that the dextrinization times should fall between 10 and 30 minutes, but up to 120 minutes or more can be used. When the hydrolyzing time is more than 30 minutes, the interval between the readings can be extended in proportion to the prolonged hydrolyzing time.

Calculation. Calculate and express results in SKB units as indicated in *Cereal Laboratory Methods* (1). Typical results are shown in Table II.

B. Rapid Procedure for End-Point Determination (Modified AACC Method IB)

To decrease the hydrolyzing time required and to reduce the risk of the bluish discoloration mentioned later, in the "Discussion," modify the mixture for end-point determination as follows: mix 3 ml. of the hydrolyzing mixture with 10 ml. of an iodine solution containing 43 g. potassium iodide and 4.3 ml. stock iodine per 1,000 ml. Determine the end-point as described in Method IA. Calculate as indicated in *Cereal Laboratory Methods* (1) and multiply by 0.7 (empirically found factor) to express the values in SKB units. Typical results obtained by this method are given in Table II.

[3] The author has corresponded with manufacturers for the purpose of having erythrodextrin in a solid state available in the market.

II. Colorimetric Method

Apparatus. (1) One water bath regulated at 30°C. and one at 20°C.[4]
(2) An instrument[5] capable of measuring absorbancy at 575 mμ.

Reagents. The reagents are the same as used in the Modified AACC Method IA, with the following exceptions:

(1) Dilute iodine solution. Dissolve 43 g. potassium iodide in water, add 4.3 ml. stock iodine solution, and make up to 1,000 ml. This solution should be at 20° or 30°C. when color comparisons are made. Make fresh solution daily.

(2) Blank for zero-point (BO). Mix 2 ml. of 0.2% calcium chloride solution, 10 ml. dilute iodine solution, and 40 ml. distilled water.

(3) Blank for starting-point (BS). Mix 10 ml. of dilute iodine solution, 40 ml. of distilled water, and 2 ml. of a mixture consisting of 1 part buffered beta-amylase limit dextrin solution and 3 parts of 0.2% calcium chloride solution. The BO and BS blanks described here have approximately the same absorbancies as corresponding mixtures containing flour extract. If individual BS blanks are prepared with each flour, the flour extract must be mixed with iodine solution before the limit dextrin is added.

Preparation of Extract and Hydrolyzing Mixture. Same as for Modified AACC Method IA.

Determination of "Period of Half-Life."[6] After mixing and attempering the BO and BS solutions exactly to 20° or 30°C., adjust the colorimeter so that the BO solution gives an absorbancy of 0 at 575 mμ, then make an absorbancy reading of the BS solution. After three or more appropriate hydrolyzing times (e.g. 10, 20, and 30 minutes), add from a fast-flowing pipet 2 ml. of the hydrolyzing mixture to solutions of 10 ml. dilute iodine solution and 40 ml. distilled water attempered exactly to 20°C., in a water bath; mix well and pour aliquots of the hydrolysate-iodine solutions into colorimeter tubes, and measure the absorbancy at 575 mμ at room temperature (about 20°C.). This makes it possible to start hydrolyzing several samples at intervals of 1 or 2 minutes. Five or ten samples can be tested in the same series. For flour with very low amylase activity it may be advisable to use more extended hydrolyzing times — e.g., 20, 40, and 60 minutes.

To prevent errors in absorbancy determinations it is important that the mixture is at 20°C., that the photometer cells (tubes) are clean, and that the readings are made immediately or preferably 10 minutes after the first mixing. Repeated mixing must also be made immediately before the absorbancy reading, and the reading should be made immediately after the cell has been inserted in the photometer.

Calculation of Alpha-Amylase Activity. For accuracy the concentration of the flour extract should be so adjusted that the absorbancy values used for calculation correspond to a "starch" conversion of 35 to 65%. For convenience and accuracy the hydrolyzing time should fall between 10 and 40 minutes (preferably 20 minutes). With a flour of low alpha-amylase activity 120 minutes or more can be used.

The absorbancy reading for BS (E_o = the absorbancy after 0 minutes) and the readings after three different times (E_t = the absorbancy after t minutes) should fall on a straight line when plotted on semilogarithmic pa-

[4] The temperature of most European laboratories is about 20°C.; that of American laboratories is about 30°C. The bath temperature selected for attempering the color-producing reagents will depend upon the laboratory temperature.

[5] A suitable inexpensive instrument is the "EEL" Colorimeter, Model A, Evans Electro-Selenium Limited, Harlow, England, using yellow filter No. 626, and standardized large tubes 5/8 in. in diameter (16 mm.), capacity 8 ml. The Spectronic "20" Colorimeter, Bausch & Lomb, using standardized ½ by 4-in. tubes, should give similar results but it has not been tested by the author.

[6] The time required for one-half of the erythrodextrin to be digested by the alpha-amylase.

per, whereupon the "period of half-life" is evaluated from the intersection of this line and a line (horizontal) for half of the absorbancy for BS ($=\frac{E_o}{2}$).

The period of half-life ($t_{\frac{1}{2}}$) in minutes can also be calculated from the formula:

$$t_{\frac{1}{2}} = t\frac{\log E_o - \log\frac{E_o}{2}}{\log E_o - \log E_t} = t\frac{0.30103}{\log E_o - \log E_t}$$

For accuracy calculate $t_{\frac{1}{2}}$ as the mean value based on two or preferably three readings following the equation for a first-order reaction.

Calculate the H unit as indicated in *Cereal Laboratory Methods* (1), using $t_{\frac{1}{2}}$ instead of "dextrinization time."

Tests performed have shown (Figs. 3 and 4) that:

One H unit $\times$ 0.42 = one SKB unit.

III. Rapid Colorimetric Method

This method, which is suitable for routine work, is similar to Method II, with the following changes:

Preparation of Extract. The extraction is limited to 5 minutes in a Waring Blendor (14,000 r.p.m.) with a temperature in the mixture after stirring of about 45°C.

Calculation of Alpha-Amylase Activity. Appropriate substitutions of data are made in the following equation for a first-order reaction:

$$V_1 = \frac{\text{"starch" (g)} \times (\log E_o - \log E_t)}{\text{flour equivalent (g)} \times \text{time (minutes)}}$$

When the relation of amount of "starch" and flour in the hydrolyzing mixtures is constant, the formula is simplified accordingly.

Tests performed have shown (Figs. 3 and 4) that:

$V_1 \times 0.82$ = SKB units.

IV. Mixture-Value Method

When the amylase activity of a sample is very low it requires a comparatively long time to reach the end-point, especially with the AACC method (1). Therefore, it can be convenient to determine the activity of a mixture containing appropriate amounts of a flour (preferably extract) of a sample of known and comparatively high alpha-amylase, and the sample with low unknown activity. The AACC method or any of the methods described above can be used to determine alpha-amylase activity of such a mixture. From these data the activity of the unknown sample can be calculated.

Example:

A = alpha-amylase activity of sample (a) in SKB units;

0.5 = alpha-amylase activity of sample (b) in SKB units;

0.095 = alpha-amylase activity of a mixture of 90% of (a) and 10% of (b);

$$A \times 0.9 + 0.5 \times 0.1 = 0.095;$$

$$A = 0.05 \text{ SKB units}$$

Thus, the mixture requires only 52.5% of the time required for sample (a) to reach the end-point. This procedure is often useful in practice and is more rapidly carried out than the method of Kneen, Sandstedt, and Miller (7), which involves prehydrolysis (about 18 hours) of the extract of the sample of low unknown activity, followed by a second hydrolysis after the addition of a known amount of alpha-amylase.

Results and Discussion

Preparation of Extract: Stone (17) and Jongh (6) used salt-solutions to extract alpha-amylase from flour. Stone (17) used an extraction time of 15 minutes with intensified stirring and Olered (12) extracted 3 to 5 minutes in a "Turmix."[7] The author has tested rapid extraction procedures (5, 10, or 15 minutes) using different stirring or revolving devices, and has found that stirring 5 minutes in a Waring Blendor can be used for rapid tests. However, the 1-hour extraction period was used in all but routine tests.

The use of highly concentrated extracts should be avoided, since they may cause a bluish discoloration which interferes with the end-point determination, especially in Method IA, where a maximum of 4 g. of wheat flour and 3 g. of rye flour should be employed. The absorbancy readings can also be influenced if the degree of starch conversion is higher than 65%.

Hydrolyzing temperatures over 30°C. Alpha-amylase activity can be determined more rapidly at temperatures higher than 30°C. (Table

TABLE II

APPROXIMATE TIME REQUIRED FOR DETERMINING THE ALPHA-AMYLASE ACTIVITY OF FLOURS BY DIFFERENT METHODS, WHEN USING A FLOUR EXTRACT CONCENTRATION OF 5 G. TO 100 ML. OF SOLUTION

Alpha-Amylase Activity	Hydrolyzing Temperature 30°C.				Hydrolyzing Temperature 40°C. Colorimetric Method II
	AACC Method[a]	Modified AACC Method 1A	Modified AACC Method 1B	Colorimetric Method II	
SKB units	*minutes*[b]	*minutes*	*minutes*	*minutes*	*minutes*
0.05	960	160	114	67	34
0.10	480	80	57	33.5	17
0.50	96	16.0	11.4	6.7	3.4
1.00	48	8.0	5.7	3.4	1.7
2.00	24	4.0	2.8	1.7	0.8
4.00	12	2	1.4	0.9	0.4
8.0	6	1	0.7	0.4	0.2

[a] See reference 1.
[b] Time to reach "end point."

[7] A high-speed stirrer manufactured by AB Turmix, Sveavägen 13, Stockholm, Sweden.

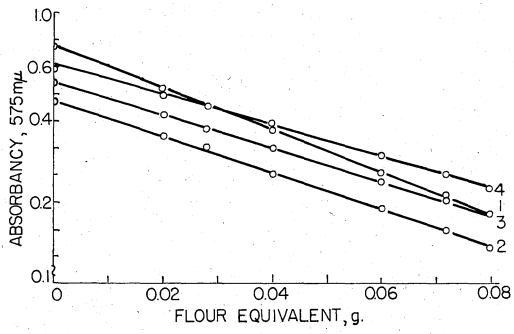

Fig. 1. Influence of composition of iodine solution on absorbancy values after varying degrees of "starch" hydrolysis during 40 minutes:

Curve	Hydrolyzing Mixture		Mixture for Absorbancy Measurements			
	"Starch" Employed (Conc. = 2%)	*Flour Extract (Conc. = Varying)*	*Hydrolysate*	*Iodine Solution (Diluted)*	*Iodine/ "Starch"*	*Potassium Iodide/ "Starch"*
	ml	*ml*	*ml*	*ml*	*mg/mg*	*mg/mg*
1	20	40	2	50	0.066	30
2	20	40	2	100	0.66	300
3	20	40	2	100	0.30	0.60
4	20	40	2	100	0.50	1.00

II). However, in evaluating the "end-point" with the Hellige disk, the hydrolysate-iodine mixture should have a temperature of 30°C.

End-Point Determination. One way to decrease hydrolyzing time is to increase the flour/erythrodextrin ratio. In Modified AACC Method IA, the amount of "starch" originally present and the concentration of iodine in the mixture for determining the end-point is the same as in the AACC method (1). In Method IB the amount of "starch" originally present in the mixture for the end-point determination is lower than in the AACC method (1) but it is still high enough to make color determination possible. In both Methods II and III more than 1 ml. of the hydrolyzing mixture has been used for the color determination for the purpose of improving reproducibility.

Especially for flours with low alpha-amylase activity, the AACC method (1) requires extremely long hydrolyzing time compared with the other methods (Table II).

Temperature of Solutions at Time of Absorbancy Readings. The temperature of the solutions for absorbancy readings is of great importance. Tests have shown that the factors for transforming H units and V_1 values into SKB units change from 0.42 to 0.33 and from 0.82 to 0.64, respectively, when the temperature of the solutions is increased from 20° to 30°C. Since a common room temperature is about 20°C., this temperature was chosen for the absorbancy readings in this study.

Evaluation of Absorbancy Readings. Since the absorbancy values are influenced by the amount of iodine and potassium iodide present in the mixture as well as the degree of dilution, tests were performed to determine which conditions were most suitable. In these tests, summarized in Fig. 1, varying degrees of "starch" conversion have been attained by using different concentrations of flour extract and the same hydrolyzing time. Because of the results obtained in these and other similar investigations, 0.0946 mg. iodine and 43.19 mg. of potassium iodide per 1 mg. "starch" employed in the hydrolyzing mixture were adopted for use in the colorimetric methods. The degree of dilution is also important and the dilution used was 40 ml. of water added to 10 ml. of diluted iodine solution and 2 ml. of hydrolysate, giving an absorbancy for BS ($= E_0$) of about 5.8 with the use of the Evans EEL Colorimeter.

A zero-order relationship was found by Hoskam (5) for the action of alpha-amylase up to 50% conversion and by Jongh (6) up to 20% conversion of erythrodextrin. With the amount of iodine solution used in the colorimetric method, a first-order reaction was found (Fig. 2) from 35 to 70% erythrodextrin conversion. Whether the reaction

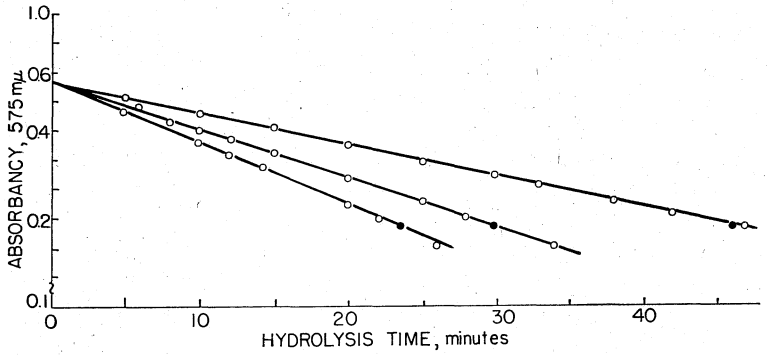

Fig. 2. Relation between absorbancy and time of hydrolysis by extracts of three different flours. Solid circles represent absorbancy at the end-point determined by Modified AACC Method IB.

proceeds according to the zero-order law with the concentration used and if so, how far, was not investigated in this work, since the absorbancy values corresponding to a starch conversion of less than 35% often were not reproducible. The absorbancy values corresponding to an erythrodextrin conversion of over 65 to 70% also were less reliable. Flour extracts which were not clear gave higher absorbancy values than expected, apparently because starch particles present in the flour extract influenced the readings.

Amylase Activity Values. The amylase activity values of wheat and rye flour or finely pulverized grains determined by the different meth-

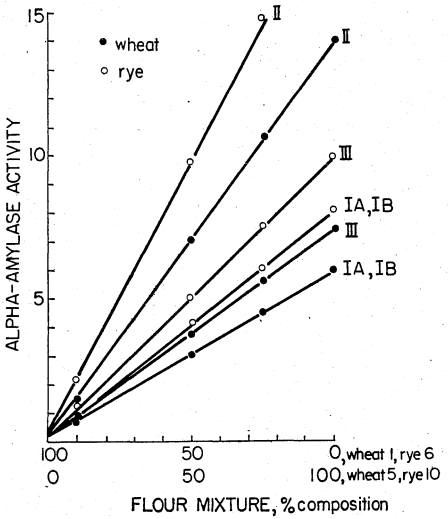

Fig. 3. Relation between alpha-amylase activity and composition of wheat and rye flour mixtures, respectively, determined by different methods. In Methods IA and IB, alpha-amylase activity is expressed in SKB units by multiplying the experimental values by the factors 1.0 and 0.7 respectively. In Method II, amylase activity is expressed in H units and in Method III, in V units.

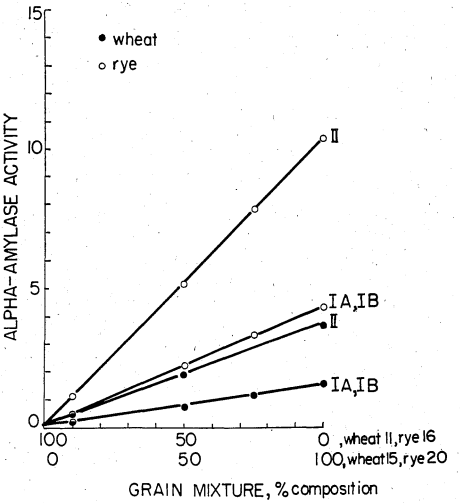

Fig. 4. Relation between alpha-amylase activity and composition of wheat and rye pulverized grain mixtures determined by different methods. In Methods IA and IB, alpha-amylase activity is expressed in SKB units by multiplying the experimental values by the factors 1.0 and 0.7 respectively. In Method II, amylase activity is expressed in H units.

ods are recorded in Figs. 3 and 4 respectively. From these and other tests the factors for transforming the values obtained into SKB units have been computed.

The reproducibility with Modified AACC Method I is about the same as with the AACC method (1). Good reproducibility also can be achieved with the colorimetric methods, especially in Method II. The values usually are evaluated from three absorbancy readings and corresponding times following the equation for a first-order reaction.

Acknowledgments

The author gratefully acknowledges the technical assistance of H. Perten, Mrs. D. Ljung, Mrs. E. Ziegler, and Mrs. J. Lunin.

The author is also indebted to Byron S. Miller, Kansas State University, Manhattan, Kansas, for assistance in revising this manuscript.

Literature Cited

1. American Association of Cereal Chemists. Cereal laboratory methods (6th ed.). The Association: St. Paul, Minnesota (1957).
2. Bawden, R. F., and Artis, W. G. Use of a photoelectric photometer to obtain the end point in the alpha-amylase determination. Am. Soc. Brewing Chemists, Proc. 1951, pp. 41–44.
3. Hagberg, S. Diastasmethoden für Weizen und Roggenmehlen. Proc. Intern. Assoc. Cereal Chemistry, Wien (1958).
4. Hanes, C. S., and Cattle, Margaret P. Starch-iodine coloration as an index of differential degradation by the amylases. The Low Temperature Research Station, Cambridge (1938).
5. Hoskam, E. G. Photometric determination of alpha- and beta-amylase in flour. Biochim. et Biophys. Acta **1:** 419–427 (1947).
6. Jongh, G. Colorimetrische bepaling van alfa-amylase. Chem. Weekblad **53:** 597–602 (1957).
7. Kneen, E., Miller, B. S., and Sandstedt, R. M. The influence of temperature on the development of amylase in germinating wheat. Cereal Chem. **19:** 11–27 (1942).
8. Kneen, E., and Sandstedt, R. M. Beta-amylase activity and its determination in germinated and ungerminated cereals. Cereal Chem. **18:** 237–252 (1941).
9. Kneen, E., Sandstedt, R. M., and Hollenbeck, C. M. The differential stability of the malt amylases — separation of the alpha and beta components. Cereal Chem. **20:** 399–423 (1943).
10. Knight, R. A. Detection and estimation of fungal amylases in flour. J. Sci. Food Agr. **11:** 54–60 (1960).
11. Lemmerzahl, J. Die Dextrinmethode zur Bestimmung der Höhe der Auswuchsschädigung bei Getreidemehlen. Brot u. Gebäck **8:** 139–141 (1955).
12. Olerod, R. Eine kinetische Methode zur Bestimmung der Alpha-Amylaseaktivität in Brotgetreide. Getreide u. Mehl **9:** 49–52 (1959).
13. Olson, W. J., Evans, Ruth, and Dickson, A. D. A modification of the Kneen and Sandstedt methods for the determination of alpha- and beta-amylases in barley malt. Cereal Chem. **21:** 533–539 (1944); **24:** 299–300 (1947).
14. Redfern, S. Methods for determination of alpha-amylase. IV. A glass end point color standard for use in the dextrinizing method; effect of temperature and starch lot on this method. Cereal Chem. **24:** 259–268 (1947).
15. Ritter, K. Über ein einfaches Verfahren zur Feststellung des Auswuchsgrades. Zeitschr. ges. Getreidew. No. 2 (1942).
16. Sandstedt, R. M., Kneen, E., and Blish, M. J. A standardized Wohlgemuth procedure for alpha-amylase activity. Cereal Chem. **16:** 712–723 (1939).
17. Stone, I. A sensitive method for the determination of alpha-amylase activity of flour. Cereal Chem. **29:** 228–234 (1952).