TC 34

INTERNATIONAL STANDARD ISO 3983

INTERNATIONAL ORGANIZATION FOR STANDARDIZATION •МЕЖДУНАРОДНАЯ ОРГАНИЗАЦИЯ ПО СТАНДАРТИЗАЦИИ•ORGANISATION INTERNATIONALE DE NORMALISATION

Cereals and cereal products — Determination of alpha-amylase activity — Colorimetric method

Céréales et produits céréaliers — Détermination de l'activité alpha-amylasique — Méthode colorimétrique

First edition — 1977-09-01

UDC 633.1 : 543.432 **Ref. No. ISO 3983-1977 (E)**

Descriptors : cereal products, grains (food), chemical tests, determination, enzymatic activity, amylase, colorimetric analysis.

Price based on 5 pages

FOREWORD

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International Standard ISO 3983 was developed by Technical Committee ISO/TC 34, *Agricultural food products,* and was circulated to the member bodies in November 1975.

It has been approved by the member bodies of the following countries :

Australia
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Romania
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Spain
Thailand
Turkey
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U.S.S.R.
Yugoslavia

No member body expressed disapproval of the document.

This International Standard is based on Standard No. 108 of the International Association for Cereal Chemistry (ICC).

Printed in Switzerland

Cereals and cereal products — Determination of alpha-amylase activity — Colorimetric method

1 SCOPE AND FIELD OF APPLICATION

This International Standard specifies a colorimetric method for the determination of alpha-amylase activity of cereals and cereal products ranging from very low to very high in alpha-amylase activity. The method may also be used for estimating the alpha-amylase activity of additives of fungal and bacterial origin.

2 REFERENCES

ISO/R 712, *Cereals and cereal products — Determination of moisture content (Routine method).*

ISO/R 950, *Cereals — Sampling (as grain).*

ISO 1666, *Starch — Determination of moisture content — Oven-drying methods.*

ISO 2170, *Cereals and pulses — Sampling of milled products.*

3 DEFINITION

The **alpha-amylase activity of a product** is equal to 1 unit if the enzyme extracted from 1 g of product in a volume of 1 litre causes, under the specified conditions, hydrolytic degradation of limit dextrin substrate of $1{,}024 \times 10^{-5}$ unit per unit substrate present per second. Limit dextrin is the product of exhaustive degradation of starch by beta-amylase.

4 PRINCIPLE

The enzyme is allowed to degrade a limit dextrin substrate. After various reaction times, aliquots of the reaction mixture are added to an iodine solution. The decrease of intensity of the colour obtained with increasing reaction times is a measure of enzyme activity.

5 REAGENTS

The reagents shall be of recognized analytical quality. The water used shall be distilled water, or water otherwise obtained of at least equivalent purity.

5.1 **Iodine,** stock solution.

Dissolve 11,0 g of potassium iodide in a minimum of water and add 5,50 g of iodine crystals. Stir until the iodine is dissolved and dilute to 250 ± 0,5 ml. Store the solution in darkness in a brown bottle. The solution may be kept for 1 month.

5.2 **Iodine,** dilute solution.

Dissolve 40,0 g of potassium iodide in water, add 4,00 ml of the stock solution (5.1) and dilute to 1 litre. Prepare the solution on the day of use.

5.3 **Buffer solution.**

Dissolve 120 ml of glacial acetic acid and 164 g of anhydrous sodium acetate or 272 g of sodium acetate trihydrate ($CH_3COONa.3H_2O$) in water and dilute to 1 litre.

5.4 **Calcium chloride,** 2 g/l solution.

Dissolve 2,0 g of anhydrous calcium chloride or 2,6 g of calcium chloride dihydrate ($CaCl_2.2H_2O$) or 3,9 g of calcium chloride hexahydrate ($CaCl_2.6H_2O$) in water and dilute to 1 000 ± 1 ml.

5.5 **Limit dextrin** solution.

Use soluble Lintner starch, special for diastatic power determination.[1] Determine its moisture content according to ISO 1666, method 1.

Use special cereal beta-amylase powder free from alpha-amylase.[2]

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[1] The following Lintner starches, equivalent to the former No. 09903 of the catalogue of Merck and Co., Rahway, N.V., U.S.A., may be used :

- J.T. Baker Chemical Co., Phillipsburg, N.J., U.S.A., No. 599.5120 of the catalogue;
- BDH Chemicals Ltd., Poole, England, No. 30260 of the catalogue;
- E. Merck, Darmstadt, RFA, No. 1252 of the catalogue.

[2] Beta-amylase available from Wallerstein Co., Staten Island 3, N.Y., U.S.A., or material of equivalent quality, may be used.

Make a suspension of an amount of starch that corresponds to 10,00 g of dry matter in approximately 20 ml of water, and add the suspension slowly, with stirring, to a beaker containing about 300 ml of boiling water. Rinse, using a wash bottle, to transfer all of the starch. Boil the solution slowly, while stirring, for 2 min and then cool it in running water until below 30 °C; cover the beaker with a watch glass to avoid formation of a "skin" on the surface of the starch solution. Add 25 ml of the buffer solution (5.3) and 250 mg of beta-amylase, dissolved in approximately 5 ml of water. Make up the volume to 500 ml, add a few drops of toluene, and shake the solution. The pH of the solution should be 4,7 ± 0,1. If it is outside of this range, the solution should be rejected.

Store the solution at room temperature for at least 20 h before use as substrate. The solution shall be stored at a temperature not exceeding 25 °C, and not for longer than 5 days.

6 APPARATUS

Usual laboratory apparatus and glassware, and :

6.1 Constant-temperature bath regulated at 30 ± 0,1 °C.

6.2 Constant-temperature bath regulated at 20 ± 0,5 °C.

6.3 Spectrophotometer capable of operating at 575 nm, or **colorimeter** provided with a yellow filter having a wavelength of maximum transmission at approximately 575 nm and of appropriate bandwidth, with a logarithmic scale expressing absorbance (optical density).

6.4 Stop-watch or **stop-clock** with a sweep seconds hand.

6.5 Sieve, with 1,0 mm wide slots (for grain).

6.6 Sieve, with 0,8 mm apertures.

6.7 Laboratory mill, preferably a hammer type (for grain).

6.8 Centrifuge (recommended for rye), fitted with cups of 100 ml capacity and capable of generating a relative centrifugal acceleration of 1 000 to 2 000 × *g*.

6.9 pH meter with glass electrode, standardized at about pH 4 and checked at about pH 10 using appropriate buffer solutions.

7 LABORATORY SAMPLE

A representative sample shall be taken according to ISO/R 950 or ISO 2170.

8 TEST SAMPLE

8.1 Preparation

8.1.1 *Grain*

The quantity of grain to be ground (pulverized) shall be at least 300 g. Pick out coarse impurities by hand; remove dust and other fine impurities by sieving using the slot sieve (6.5). Grind the material remaining on the sieve in the mill (6.7) to pass through the 0,8 mm aperture sieve (6.6) and then blend thoroughly.

8.1.2 *Flour*

Sift the flour through the 0,8 mm aperture sieve (6.6) to break up lumps.

8.2 Moisture content

Determine the moisture content of the test sample according to ISO/R 712.

9 PROCEDURE

9.1 Adjustment of spectrophotometer or colorimeter

Pipette into a 100 ml beaker 2,0 ml of the calcium chloride solution (5.4) and 10,0 ml of the dilute iodine solution (5.2). Add from a burette the volume of water that has been found necessary by previous test (see 9.2) and mix. Bring to 20 °C by immersing the beaker in the water bath (6.2).

Fill a cell with some of this solution, place the cell in the spectrophotometer (adjusted to a wavelength of 575 nm) or the colorimeter (fitted with the chosen yellow filter) (6.3) and adjust the slit width so that the instrument reads zero on the absorbance scale. Check the adjustment regularly during the measurements.

9.2 Check of substrate solution

Pipette 5,0 ml of the limit dextrin solution (5.5) and 15,0 ml of the calcium chloride solution (5.4) into a 100 ml beaker, mix and pipette 2,0 ml of the mixture into another dry 100 ml beaker. Pipette 10,0 ml of the dilute iodine solution (5.2) into this beaker and add, from a burette, a certain, noted volume of water. Mix, and adjust the temperature to 20 °C by immersing the beaker in the water bath (6.2).

Fill a cell with some of this mixture, place the cell in the chosen instrument and note the absorbance reading, using a cell filled with the solution used in 9.1 as reference. By trial and error, adjust the volume of water added from the burette so that the absorbance reading is between 0,55 and 0,60 (5,5 and 6,0 on certain instruments). Note this volume of water and use it for the adjustment of the spectrophotometer or colorimeter in 9.1 and for the determination of all absorbance values in 9.4. If a different quantity has been used for adjustment of the instrument, readjust it with the correct quantity of water.

9.3 Extraction of enzyme

9.3.1 *Test portion*

Weigh in a flask or tube, fitted with a stopper, approximately 5 g of ground grain (8.1.1) or flour (8.1.2), to the nearest 0,05 g. Smaller amounts of materials with high enzyme activity may be used, provided that the precision of weighing is 1 % or better, and that the mass exceeds 2/*A* grams, where *A* is the activity of the product in units.

9.3.2 *Extraction*

Add to the test portion 100 ± 0,5 ml of the calcium chloride solution (5.4), previously brought to approximately 30 °C by placing it in the water bath (6.1), and mix throughly by shaking. Immediately after mixing, place the flask or tube in the water bath (6.1) at 30 °C. After 15, 30 and 45 min, take it from the water bath, turn it upside down and back ten times, and reimmerse it. (Continuous shaking gives different results and is not recommended.)

After 60 min of extraction, take out the flask or tube and, without shaking, immediately decant the contents into a centrifuge tube, or pour into a filter. Solutions must not be left standing in the mixing vessel. Either centrifuge the suspension for 10 min at 1 000 to 2 000 × *g* or filter a sufficient quantity for the test, rejecting the first drops until a clear extract is obtained.[1] It is recommended that rye extracts be centrifuged since they are often difficult to filter. Use the filtrate or supernatant liquid.

9.4 Determination of degradation of limit dextrin

Pipette 15,0 ml of the enzyme extract (9.3.2) into a 50 ml test tube or flask, insert the stopper and immerse in the water bath (6.1) at 30 °C. Pour approximately 20 ml of the limit dextrin substrate (5.5) into a test tube or flask, insert the stopper and immerse also in the water bath (6.1) at 30 °C.

When the extract and substrate have reached 30 °C (after immersion for 5 to 10 min), pipette (using a rapid flow pipette) 5,0 ml of the substrate into the tube or flask containing the 15,0 ml of enzyme extract, insert the stopper and mix by shaking vigorously. Simultaneously with pipetting the substrate into the extract, start the stop-watch or stop-clock (6.4).

Pipette into each of several 50 ml test tubes or flasks 10,0 ml of the dilute iodine solution (5.2), add from a burette the volume of water noted in the check of the substrate solution (9.2), mix by swirling, insert the stoppers and immerse in the water bath (6.2) at 20 °C.

At intervals of 5 or 10 min take the following actions :

a) pipette 2,0 ml of the enzyme-substrate mixture into one of the test tubes or flasks containing the mixture of dilute iodine solution and water;

b) mix by swirling;

c) bring to 20 °C by immersion in the water bath (6.2);

d) pour into a cell and read the absorbance as in 9.2.

The temperature of the solution in the spectrophotometer or colorimeter affects the reading of the absorbance and should, therefore, be maintained at 20 °C. The time-interval between pipetting and absorbance reading normally has no immediate influence on the results, but it should not exceed 1 h. The measurement of the absorbance of a series of solutions can be delayed until after the last 2 ml portion of the enzyme-substrate mixture has been pipetted.

The enzyme concentration in the reaction mixture should be adjusted, if possible, so that 35 to 60 % of the substrate is degraded within 15 to 40 min, i.e. the last absorbance reading should be 40 to 65 % of that of the check of the substrate solution (9.2). If the absorbance decreases too rapidly, dilute the enzyme extract with calcium chloride solution (5.4) and carry out a new determination. If the amylase activity of the sample is low, as may be the case with some flour samples, it will be necessary to extend the reaction time to 60 min or more to obtain accurate readings.

NOTES

1 Whether the degradation of substrate at a certain time is within the range of 35 to 60 % can easily be established without use of the instrument by means of visual comparison. Mix 1 part of the limit dextrin substrate solution (5.5) with 3 parts of the calcium chloride solution (5.4). Prepare two mixtures of 10 ml of the dilute iodine solution (5.2) and the volume of water noted in the check of the substrate solution (9.2). Add to one of these mixtures 1,3 ml and to the other 0,8 ml of the mixture of limit dextrin and calcium chloride solution. The colour intensities obtained correspond to 35 and 60 % degradation, respectively. Colour intensities from aliquots of the enzyme-substrate mixture can be compared visually with these two matching solutions.

2 In all measurements, the ratio between the volume of enzyme extract, or diluted enzyme extract, or calcium chloride solution [in the check of the substrate solution (9.2)], and that of the limit dextrin substrate must be 3 : 1.

10 EXPRESSION OF RESULTS

10.1 Method of calculation and formula

10.1.1 The alpha-amylase activity is given by the equation

$$A = \frac{500 \times f}{m} \times \frac{100}{100 - h} \times \frac{\log_{10} D_1 - \log_{10} D_2}{t_1 - t_2}$$

$$= \frac{500 \times f \times b}{m} \times \frac{100}{100 - h} \qquad \ldots (1)$$

where

A is the alpha-amylase activity on the dry basis, in units;

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[1] A suitable filter paper is Whatman No. 42.

m is the mass, in grams, of product extracted with 100 ml of calcium chloride solution (9.1);

h is the moisture content, in per cent;

f is the dilution factor if the enzyme extract has been diluted before the addition of substrate (9.4);

D_1 and D_2 are the absorbances (optical densities) corresponding to the time-intervals t_1 and t_2;

t_1 and t_2 are the time-intervals, in minutes, between the addition of substrate to the enzyme, and pipetting of this mixture into the iodine solution (9.4);

b is the (absolute value of the) slope of a plot of $\log_{10} D$ against t.

NOTES

1 The unit defined above is called A-unit. It has been established empirically that 1 A-unit is equivalent to 0,11 SKB-units. See H. Perten, *Cereal Chem.* **43**, pp. 336-342 (1966).

2 The factor 500 in equation (1) is introduced in order to avoid too low figures for normal flours. Its correspondence to the definition in clause 3 is demonstrated by the following argument.

The enzyme concentration during the determination, in grams of dry product per litre, corresponds to

$$m \times \frac{100-h}{100} \times \frac{1\,000}{100} \times \frac{15}{15+5} \times \frac{1}{f} = 7{,}5 \times \frac{m}{f} \times \frac{100-h}{100}$$

Since the absorbance reading, D, is proportional to the amount of substrate, S, application of the definition leads to

$$b = -\frac{\mathrm{d}\log_{10} D}{\mathrm{d}t} = -\frac{1}{2{,}303 \times S} \times \frac{\mathrm{d}S}{\mathrm{d}t}$$

$$= 1{,}024 \times 10^{-5} \times \frac{60}{2{,}303} \times 7{,}5 \times \frac{m}{f} \times \frac{100-h}{100} \times A$$

$$= 2{,}00 \times 10^{-3} \times \frac{m}{f} \times \frac{100-h}{100} \times A \quad \ldots (2)$$

The factor 60 is introduced since t is expressed in minutes. Equation (2) is equivalent to equation (1).

The rate of the enzymic degradation of the limit dextrin is characterized by the ratio $(\log_{10} D_1 - \log_{10} D_2)/(t_2 - t_1)$. This ratio can be estimated from pairs of observations; usually the first absorbance reading after 5 or 10 min is chosen as D_1, but other observations can also be used for this purpose. Alternatively, this ratio can be estimated from a larger number of observations by taking the slope b of a plot of $\log_{10} D$ against t.

Example

A 5,20 g sample with 14,60 % moisture was extracted with 100 ml of calcium chloride solution. Since the rate of the reaction with limit dextrin was too high, the enzyme extract was diluted with calcium chloride solution. To 1 part of the extract, 1,5 parts of the calcium chloride solution were added. Therefore, $f = 1 + 1{,}5 = 2{,}5$.

After time-intervals of 5, 10 and 20 min after mixing the substrate and enzyme extract, the absorbance readings were :

Time min	**Absorbance**	$\log_{10} D$ + 1
5	0,498	0,697
10	0,425	0,628
20	0,308	0,489

From the observations after 5 and 10 min,

$$b = \frac{0{,}697 - 0{,}628}{10 - 5} = 0{,}013\,8\ \mathrm{min}^{-1}$$

From a plot of $\log_{10} D$ against t (see figure), using all three observations,

$$b = 0{,}013\,91\ \mathrm{min}^{-1}$$

Substitution of the latter result into equation (1) leads to

$$A = \frac{500 \times 2{,}5 \times 0{,}013\,91}{5{,}20} \times \frac{100}{100 - 14{,}60}$$

$$= 3{,}9 \text{ units}$$

10.1.2 Report the mean value of the results of two determinations, provided that the difference between these two results is smaller than the maximum indicated in 10.2. If this difference is larger, repeat the determination in duplicate and report the mean value of the new results, provided that their difference meets the requirements in 10.2.

Round off according to the following table :

Activity units	**Rounding-off interval** units
< 50	0,1
50 to 500	1
500 to 5 000	10
5 000 to 50 000	100

etc.

10.2 Repeatability

The difference between the results of two determinations carried out simultaneously or in rapid succession in the same laboratory shall not exceed 10 % of their mean value or, if this mean value is less than 2 units, 0,2 unit.

11 TEST REPORT

The test report shall show the method used and the result obtained. It shall also mention any operating conditions not specified in this International Standard, or regarded as optional, as well as any circumstances that may have influenced the result.

The report shall include all details required for complete identification of the sample.