INTERNATIONAL STANDARD

ISO 13965

First edition
1998-09-15

Meat and meat products — Determination of starch and glucose contents — Enzymatic method

Viande et produits à base de viande — Détermination des teneurs en amidon et en glucose — Méthode enzymatique

Foreword

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Draft International Standards adopted by the technical committees are circulated to the member bodies for voting. Publication as an International Standard requires approval by at least 75 % of the member bodies casting a vote.

International Standard ISO 13965 was prepared by Technical Committee ISO/TC 34, *Agricultural food products*, Subcommittee SC 6, *Meat and meat products.*

Annexes A and B of this International Standard are for information only.

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Meat and meat products — Determination of starch and glucose contents — Enzymatic method

1 Scope

This International Standard specifies an enzymatic method for the determination of water-free starch content and glucose content of all kinds of meat and meat products, including poultry.

The method is suitable for the quantitative determination of starch and glucose contents down to levels of 0,30 % (*m/m*).

The method is not applicable for chemically modified starches or their derivatives.

2 Normative reference

The following standard contains provisions which, through reference in this text, constitute provisions of this International Standard. At the time of publication, the edition indicated was valid. All standards are subject to revision, and parties to agreements based on this International Standard are encouraged to investigate the possibility of applying the most recent edition of the standard indicated below. Members of IEC and ISO maintain registers of currently valid International Standards.

ISO 3696:1987, *Water for analytical laboratory use — Specification and test methods.*

3 Definitions

For the purposes of this International Standard, the following definitions apply.

3.1
starch content of meat and meat products
starch content determined in accordance with the procedure described in this International Standard, and expressed as a percentage by mass

3.2
glucose content of meat and meat products
glucose content determined in accordance with the procedure described in this International Standard, and expressed as a percentage by mass

4 Principle

4.1 Hydrolysis of the starch present in a test portion with the enzyme α-amylase at pH = 5,0 for 15 min. Determination of the starch content using the following enzymatic reactions.

4.2 Hydrolysis of the solubilized starch to yield glucose using amyloglucosidase (AGS):

$$\text{starch} + (n-1)H_2O \overset{AGS}{\rightleftharpoons} \text{glucose}$$

For the determination of the glucose content, this step is omitted.

4.3 Phosphorylation of the glucose generated by means of adenosine 5'-triphosphate (ATP) to yield glucose 6-phosphate (G-6-P) using hexokinase (HK):

$$\text{glucose} + \text{ATP} \overset{HK}{\rightleftharpoons} \text{glucose 6-phosphate} + \text{ADP}$$

4.4 Oxidation of glucose 6-phosphate (G-6-P) by means of nicotinamide adenine dinucleotide phosphate (NADP) to gluconate 6-phosphate using glucose 6-phosphate dehydrogenase (G-6-PDH):

$$\text{glucose 6-phosphate} + NADP^+ \overset{G\text{-}6\text{-}PDH}{\rightleftharpoons} \text{gluconate 6-phosphate} + NADPH + H^+$$

4.5 Spectrometric measurement of the amount of reduced nicotinamide dinucleotide phosphate (NADPH) at a wavelength of 340 nm.

5 Reagents

Use only reagents of recognized analytical grade, unless otherwise specified.

5.1 Water, complying with at least grade 3 in accordance with ISO 3696.

5.2 α-Amylase (EC 3.2.1.1), enzyme suspension.

A liquid enzyme preparation of a heat-stable α-amylase produced from *Bacillus licheniformis* (Termamyl® 120L)[1].

5.3 Sodium hydroxide solution, c(NaOH) = 5 mol/l.

Dissolve 200 g of sodium hydroxide in water. Cool to room temperature, dilute to 1000 ml and mix.

5.4 Sodium hydroxide solution, c(NaOH) = 0,5 mol/l.

Dissolve 20 g of sodium hydroxide in water. Cool to room temperature, dilute to 1000 ml and mix.

5.5 Ammonium sulfate solution, $c[(NH_4)_2SO_4]$ = 3,2 mol/l.

Dissolve 422 g of ammonium sulfate in water. Dilute to 1000 ml and mix.

5.6 Acetate buffer, $c(CH_3CO_2Na)$ = 0,1 mol/l, pH = 5,0.

Dissolve 6,80 g of sodium acetate trihydrate ($CH_3CO_2Na \cdot 3H_2O$) in 400 ml of water. Adjust the pH to 5,0 with hydrochloric acid or sodium hydroxide solution with a pH-meter (6.2). Dilute with water to 500 ml and mix.

The solution is stable for at least 3 months at +4 °C in the dark.

5.7 Citrate buffer, c(citrate) = 0,05 mol/l, pH = 4,6.

Dissolve 440 mg of citric acid monohydrate ($C_6H_8O_7 \cdot H_2O$) and 850 mg of trisodium citrate dihydrate ($C_6H_5Na_3O_7 \cdot 2H_2O$) in water. Dilute with water to 100 ml and mix. Check the pH with a pH-meter (6.2) and adjust if necessary with hydrochloric acid or sodium hydroxide solution.

The solution is stable for at least 3 months at +4 °C in the dark.

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1) Termamyl® 120 L is an example of a suitable product available commercially from Novo, Denmark, and Tecator, Sweden. This information is given for the convenience of users of this International Standard and does not constitute an endorsement by ISO of this product.

5.8 Triethanolamine buffer, c(triethanolamine) = 0,75 mol/l, pH = 7,6.

Dissolve 14,0 g of triethanolamine hydrochloride ($C_6H_{15}NO_3$·HCl) and 0,25 g of magnesium sulfate heptahydrate ($MgSO_4$·$7H_2O$) in 80 ml of water. Set the pH at 7,6 with a pH-meter, using the sodium hydroxide solutions (5.3 and 5.4). Dilute with water to 100 ml and mix.

The solution is stable for 4 weeks at +4 °C in the dark.

5.9 Nicotinamide adenine dinucleotide phosphate solution, c(β-NADP-Na_2) = 12,7 x 10^{-3} mol/l.

Dissolve 100 mg of NADP disodium salt in 10,0 ml of water and mix.

The solution is stable for 4 weeks at +4 °C in the dark.

5.10 Adenosine-5'-triphosphate solution, c(5'-ATP-Na_2H_2·$3H_2O$) ≈ 81 x 10^{-3} mol/l.

Dissolve 500 mg of 5'-ATP-Na_2H_2·$3H_2O$ and 500 mg of anhydrous monosodium hydrogen carbonate ($NaHCO_3$) in 10,0 ml of water and mix.

The solution is stable for 4 weeks at +4 °C in the dark.

5.11 Amyloglucosidase (AGS; EC 3.2.1.3), enzyme suspension in ammonium sulfate solution (5.5), ρ(AGS) = 10 mg/ml.

The specific activity of the enzyme shall be 14 units per milligram.

The suspension is stable for 1 year at +4 °C in the dark.

5.12 Hexokinase (HK; EC 2.7.1.1)/glucose 6-phosphate dehydrogenase (G-6-PDH; EC 1.1.1.49), enzyme suspension in ammonium sulfate solution (5.5), ρ(HK) = 2 mg/ml and ρ(G-6-PDH) = 1 mg/ml.

The specific activity of both enzymes shall be 140 units per milligram.

The suspension is stable for 1 year at +4 °C in the dark.

6 Apparatus

Usual laboratory apparatus and, in particular, the following.

6.1 Mechanical or electrical equipment, capable of homogenizing the laboratory sample.

This includes a high-speed rotational cutter, or a mincer fitted with a plate with apertures not exceeding 4,0 mm in diameter.

6.2 pH-meter.

6.3 Fluted filter papers, glucose free, of diameter about 15 cm.

6.4 Pipettes, calibrated, for enzymatic analysis, or automatic micropipettes of equivalent quality with the following volumes: 20 µl, 50 µl, 100 µl and 200 µl.

6.5 Small plastics spatula, for mixing the contents of a cuvette (6.9).

6.6 Water bath, capable of being maintained at (60 ± 2) °C.

6.7 Hot plate.

6.8 Spectrometer, capable of measuring at a wavelength of 340 nm.

NOTE When a spectrometer fitted with a mercury vapour lamp is available, the readings can be carried out at 365 nm and 334 nm. The molecular absorption coefficient κ for NADPH is 3,51 $l \cdot mmol^{-1} \cdot cm^{-1}$ at 365 nm and 6,18 $l \cdot mmol^{-1} \cdot cm^{-1}$ at 334 nm.

6.9 Cuvettes, made of quartz or glass, with lid, or disposable cuvettes for single use, made of polymethacrylate, of 10 mm optical path length.

6.10 Analytical balance, capable of weighing to the nearest 0,1 mg.

7 Sampling

Sampling is not part of the method specified in this International Standard. A recommended sampling method is given in ISO 3100-1 [1].

It is important that the laboratory receive a sample which is truly representative and has not been damaged or changed during transport or storage.

Start from a representative sample of at least 200 g. Store the sample in such a way that deterioration and change in composition are prevented.

8 Preparation of test sample

Homogenize the laboratory sample with the appropriate equipment (6.1). Take care that the temperature of the sample material does not rise above 25 °C. If a mincer is used, pass the sample at least twice through the equipment.

Fill a suitable airtight container with the prepared sample. Close the container and store in such a way that deterioration and change in composition are prevented. Analyse the sample as soon as practicable, but always within 24 h after homogenization.

9 Procedure

NOTE Muscle glycogen, which occurs normally in, for example, sausages, does not interfere with the determination. Maltose interferes because this disaccharide is hydrolysed by amyloglucosidase into glucose. Maltose (and glucose) can, however, be extracted from the sample with alcohol.

9.1 Test portion

If the test sample contains maltose, make sure that the water content does not exceed 20 % (m/m). If necessary, dry the test sample.

Weigh about 400 mg of the prepared test sample (see clause 8) to the nearest 0,1 mg into a centrifuge tube. Proceed in accordance with 9.2.

If the test sample does not contain maltose, weigh between 100 mg and 1,0 g (m) of the prepared test sample to the nearest 0,1 mg into a 100 ml conical flask.

Add 30 ml of acetate buffer (5.6) and proceed in accordance with 9.3.

9.2 Extraction of maltose (and glucose)

Wash the sample three times with 10 ml of 40 % (V/V) ethanol and centrifuge after each washing. Filter the supernatant. Combine the precipitate in the centrifuge tube with that on the filter and transfer to a 100 ml conical flask using 4 x 5 ml of acetate buffer (5.6). Add 10 ml of acetate buffer (5.6).

9.3 Preparation of extract

Pipette (6.4) 50 µl of α-amylase suspension (5.2) into the conical flask containing the sample. Cover the conical flask with aluminium foil and boil on a hot plate (6.7) for 15 min. Shake the flask at intervals.

Then keep the conical flask at 60 °C in the water bath (6.6) for 15 min. Quantitatively transfer the contents of the conical flask to a 100 ml volumetric flask. Rinse the conical flask with warm water and add the washings to the volumetric flask. Allow to cool to room temperature and dilute to the mark with water. Filter (6.3) at least 10 ml of the sample extract, discarding the first few millilitres, and immediately proceed in accordance with 9.4.

Keep samples containing fat for 1 h in the refrigerator before filtration.

9.4 Determination

9.4.1 Prepare a reagent blank solution as follows. Pipette (6.4) 200 µl of citrate buffer (5.7) and 100 µl of water into a cuvette (6.9) containing a spatula (6.5).

9.4.2 Prepare a test solution for the glucose determination as follows. Pipette (6.4) 200 µl of citrate buffer (5.7) and 100 µl (V_2) of the sample extract (9.3) into a cuvette (6.9) containing a spatula (6.5).

9.4.3 Prepare a test solution for the starch determination as follows. Pipette (6.4) 200 µl of citrate buffer (5.7) and 100 µl (V_2) of the sample extract (9.3) into a cuvette (6.9) containing a spatula (6.5).

If the concentration of starch in the sample solution exceeds 0,4 g/l, dilute it before analysis.

9.4.4 Pipette (6.4) 20 µl of AGS suspension (5.11) into the cuvette containing the reagent blank solution (9.4.1) and into the cuvette containing the test solution for the starch determination (9.4.3). Pipette 20 µl of water into the cuvette containing the test solution for the glucose determination (9.4.2).

Mix the contents of the cuvettes by swirling or by moving the spatula up and down.

Close the cuvettes with the lid or otherwise (e.g. paraffin sheet). Keep the cuvettes in the water bath (6.6) for 15 min at (60 ± 2) °C.

The above pipette procedure is schematically presented below:

Reagent	Reagent blank solution	Test solution for glucose determination	Test solution for starch determination
Citrate buffer (5.7)	200 µl	200 µl	200 µl
Sample extract (9.3)	—	100 µl	100 µl
Water (5.1)	100 µl	20 µl	—
AGS suspension (5.11)	20 µl	—	20 µl

The volume of sample extract pipetted into the cuvette may be increased up to 1,0 ml of aqueous solution. In this case, adjust the pH of the filtrate to pH = 4 to pH = 5. Accordingly reduce the volume of 1,50 ml of water to be added to the reaction mixture in 9.4.5, in order to obtain the same final volume V_1 in 9.4.6.

9.4.5 Cool the cuvettes to (22,5 ± 2,5) °C and clean their outside surfaces. Pipette (6.4) successively to all cuvettes 1,00 ml of the triethanolamine buffer (5.8), 100 µl of the NADP solution (5.9), 100 µl of the ATP solution (5.10) and 1,50 ml of water. Mix carefully by swirling or with the spatula (6.5).

Read the absorbance A_1 (6.8) of each cuvette at a wavelength of 340 nm against water after 3 min.

9.4.6 Pipette (6.4) into each of the cuvettes 20 µl of HK/G-6-PDH suspension (5.12). Mix the contents of the cuvettes by moving the spatula up and down. The final cuvette volume is 3,04 ml (V_1).

Read the absorbance A_2 of each cuvette at a wavelength of 340 nm against water after 15 min.

NOTE The reaction is normally completed within 5 min to 10 min. If the reaction does not stop within this time, repeat this reading every 2 min until a constant increase of absorbance every 2 min is detected. Extrapolate the absorbance to the time of addition of the enzyme HK/G-6-PDH (for an example, see figure A.1).

10 Calculation

10.1 Water-free starch content

10.1.1 Absorbance difference

Calculate the absorbance difference using the equation

$$\Delta A_s = \left(A_{2s} - A_{1s}\right) - \left(A_{2g} - A_{1g}\right) - \left(A_{2b} - A_{1b}\right)$$

where

ΔA_s is the absorbance difference due to the starch content;

A_{1b} is the absorbance measured in 9.4.5 of the reagent blank solution;

A_{1g} is the absorbance measured in 9.4.5 of the test solution for the glucose determination;

A_{1s} is the absorbance measured in 9.4.5 of the test solution for the starch determination;

A_{2b} is the absorbance measured in 9.4.6 of the reagent blank solution;

A_{2g} is the absorbance measured in 9.4.6 of the test solution for the glucose determination;

A_{2s} is the absorbance measured in 9.4.6 of the test solution for the starch determination.

10.1.2 Calculation of water-free starch content

Calculate the water-free starch content using the equation

$$w_s = \frac{\Delta A_s \times M_{rgs} \times V_1 \times f \times 100}{d \times \kappa \times m \times V_2 \times 1000 \times 10}$$

where

w_s is the numerical value of the water-free starch content, as a percentage by mass, of the test sample;

ΔA_s is the absorbance difference calculated in 10.1.1;

M_{rgs} is the relative molecular mass of glucose in starch ($M_{rgs} = M_{rglucose} - M_{rwater} = 162{,}1$);

V_1 is the numerical value of the final volume, in millilitres, in the cuvette (V_1 = 3,04 ml);

f is the dilution factor;

d is the numerical value of the optical path length, in centimetres, of the cuvette;

κ is the numerical value of the molar absorption coefficient, in litres per millimole centimetre, of NADPH (κ = 6,30 when measured at 340 nm);

m is the numerical value of the mass, in grams, of the test portion (9.1);

V_2 is the numerical value of the volume, in millilitres, of sample extract added in 9.4.3.

Round the result to two decimal places.

10.2 Glucose content

10.2.1 Absorbance difference

Calculate the absorbance difference using the equation

$$\Delta A_g = \left(A_{2g} - A_{1g}\right) - \left(A_{2b} - A_{1b}\right)$$

where

ΔA_g is the absorbance difference due to the glucose content;

A_{1b} is the absorbance measured in 9.4.5 of the reagent blank solution;

A_{1g} is the absorbance measured in 9.4.5 of the test solution for the glucose determination;

A_{2b} is the absorbance measured in 9.4.6 of the reagent blank solution;

A_{2g} is the absorbance measured in 9.4.6 of the test solution for the glucose determination.

10.2.2 Calculation of glucose content

Calculate the glucose content using the equation

$$w_g = \frac{\Delta A_g \times M_{rg} \times V_1 \times f \times 100}{d \times \kappa \times m \times V_2 \times 1000 \times 10}$$

where

w_g is the numerical value of the glucose content, as a percentage by mass, of the test sample;

ΔA_g is the absorbance difference calculated in 10.2.1;

M_{rg} is the relative molecular mass of glucose (M_{rg}= 180,2);

V_1 is the numerical value of the final volume, in millilitres, in the cuvette (V_1 = 3,04 ml);

f is the dilution factor;

d is the numerical value of the optical path length, in centimetres, of the cuvette;

κ is the numerical value of the molar absorption coefficient, in litres per millimole centimetre, of NADPH (κ = 6,30 when measured at 340 nm);

m is the numerical value of the mass, in grams, of the test portion (9.1);

V_2 is the numerical value of the volume, in millilitres, of sample extract added in 9.4.2.

Round the result to two decimal places.

11 Precision

11.1 Repeatability

11.1.1 Water-free starch content

The absolute difference between two independent single test results, obtained using the same method on identical test material with starch contents between 0,3 % (*m/m*) and 4,0 % (*m/m*) in the same laboratory by the same operator using the same equipment within a short interval of time, will in not more than 5 % of cases exceed the repeatability limit r_s given by the equation:

$$r_s = 0{,}102 + 0{,}132 \times \overline{w}_s$$

where

r_s is the numerical value of the repeatability limit, as a percentage by mass, for water-free starch content;

$\overline{w}_s$ is the mean of the two results for water-free starch content.