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Animal feeding stuffs — Enzymatic determination of total starch content

Aliments des animaux — Détermination enzymatique de la teneur totale en amidon

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Foreword

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International Standards are drafted in accordance with the rules given in the ISO/IEC Directives, Part 2.

The main task of technical committees is to prepare International Standards. 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.

Attention is drawn to the possibility that some of the elements of this document may be the subject of patent rights. ISO shall not be held responsible for identifying any or all such patent rights.

ISO 15914 was prepared by Technical Committee ISO/TC 34, *Food products*, Subcommittee SC 10, *Animal feeding stuffs*.

Animal feeding stuffs — Enzymatic determination of total starch content

1 Scope

This international Standard specifies a method for the enzymatic determination of the total starch content of animal feeding stuffs and raw materials for animal feeding stuffs.

The method is also applicable to the determination of the purity of starch.

It is important that in the sample matrix no components are present which contribute to the measured extinction at 340 nm.

The analytical range of the method is 40 g/kg to 1 000 g/kg starch. The standard procedure is applicable to the range 200 g/kg to 1000 g/kg. For the lower range, 40 g/kg to 200 g/kg, another dilution procedure for the standard glucose solution and samples can be used.

2 Normative references

The following referenced documents are indispensable for the application of this document. For dated references, only the edition cited applies. For undated references, the latest edition of the referenced document (including any amendments) applies.

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

ISO 6498:1998, *Animal feeding stuffs — Preparation of test samples*

3 Terms and definitions

For the purpose of this document, the following terms and definitions apply.

3.1
starch
natural vegetable polymer consisting of long linear unbranched chains of 1,4-α-D-glucose units (amylose) and/or long α-1,6-branched chains of α-1,4-linked glucose units (amylopectin)

3.2
starch content
mass fraction of starch and its high molecular mass breakdown products, insoluble in 40 % ethanol, and determined in accordance with the method of this International Standard

NOTE The starch content is expressed in grams per kilogram.

4 Principle

The milled test sample is extracted with 40 % ethanol to remove the soluble sugars. Sample disintegration and starch solubilization is achieved by dispersing the extracted test portion in aqueous DMSO (90 % volume fraction) at 100 °C, followed by addition of concentrated hydrochloric acid at 60 °C. The solubilized and dissolved starch is quantitatively converted into glucose by the enzyme amyloglucosidase. The glucose quantification is carried out by the well-known hexokinase method (see [1], [2]).

5 Reagents

Use only reagents of recognized analytical grade.

5.1 Water, complying with at least grade 3 in accordance with ISO 3696:1987.

5.2 Ethanol (C_2H_5OH), 40 % (volume fraction)

Take 417 ml of ethanol (96 % volume fraction) and dilute with water to 1 000 ml

5.3 Hydrochloric acid, $c(HCl) = 12$ mol/l.

5.4 Aqueous sodium hydroxide, $c(NaOH) = 4$ mol/l.

Dissolve in a beaker 40 g of NaOH in about 50 ml water. After cooling, transfer quantitatively to a 250 ml volumetric flask and dilute to the mark with water.

WARNING — Heat develops. Wear safety glasses.

5.5 Acetic acid solution, $c(CH_3COOH) = 2$ mol/l.

Add to a 500 ml volumetric flask about 200 ml water, followed by 59 ml of glacial acetic acid. Dilute to the mark with water.

5.6 Sodium acetate solution, $c(CH_3COONa) = 2$ mol/l.

Dissolve, in a 500 ml volumetric flask, 82,0 g of sodium acetate in water and dilute to the mark with water.

5.7 Sodium acetate buffer, $c(CH_3COONa/H) = 2$ mol/l, pH = 4,8.

Mix 41 ml of acetic acid solution (5.5) with 59 ml of sodium acetate solution (5.6). Check the pH with a pH-meter and, if necessary, adjust to obtain the correct pH with the acetic acid or the sodium acetate solution.

Prepare a fresh buffer solution daily.

5.8 Aqueous dimethylsulfoxide, (σ_{DMSO}) = 90 % (volume fraction).

Mix pure DMSO and water in a volume ratio of 9:1.

5.9 Clarifying solutions, according to Carrez, prepared as follows.

5.9.1 Potassium hexacyanoferrate(II) solution, $c[K_4Fe(CN)_6] = 0,25$ mol/l.

In a 1 litre volumetric flask, dissolve 106 g of potassium hexacyanoferrate(II) trihydrate [$K_4Fe(CN)_6 \cdot 3H_2O$] in water. Dilute to the mark with water.

5.9.2 Zinc acetate, solution in 0,5 mol/l acetic acid, $c[Zn(CH_3CO_2)_2] = 1$ mol/l.

In a 1 litre volumetric flask, dissolve 219,5 g of zinc acetate dihydrate [$Zn(CH_3CO_2)_2{\cdot}2H_2O$] and 30 g of glacial acetic acid in water. Dilute to the mark with water.

5.10 Iodine solution in potassium iodide

In a 1 litre volumetric flask, dissolve 12,7 g of iodine (I_2) and 24,0 g of potassium iodide (KI) in water. Dilute to the mark with water. Dilute this solution 10-fold before use.

5.11 Standard glucose solution

5.11.1 Samples containing 200 g/kg to 1 000 g/kg starch

Prepare three independent standard glucose solutions (c = 0,0194 mol/l). In each 100 ml volumetric flask dissolve 350 mg anhydrous glucose ($C_6H_{12}O_6$) to the nearest mg in water. Dilute to the mark with water.

5.11.2 Samples containing 40 g/kg to 200 g/kg starch

Prepare three independent standard glucose solutions (c = 0,0039 mol/l). In each 500 ml volumetric flask, dissolve 350 mg ± 1 mg of anhydrous glucose ($C_6H_{12}O_6$) in water. Dilute to the mark with water.

Prepare fresh standard glucose solutions daily.

5.12 Amyloglucosidase solution, 160 U/ml AMG.

Dissolve, in a mixture of 9 ml of water and 1 ml of sodium acetate buffer (5.7), 267 mg of amyloglucosidase (AMG) [EC 3.2.1.3 (*Aspergillus niger*; Roche Diagnostics, No. 1 202 367, 6 U/mg)].[1)] With respect to storage and the use of the enzymes, follow carefully the recommendations of the enzyme supplier.

When another enzyme is used, the activity should be estimated as given in Note 1. The mass of the enzyme should be adapted to the activity found.

NOTE 1 Different enzyme suppliers use different definitions for the units of activities of enzymes. In this International Standard the following definition for the unit of activity for AMG has been used: 1 unit of amyloglucosidase will release 1 µmol of glucose from glycogen in 1 min at 25 °C and pH = 4,75.

NOTE 2 10 ml of enzyme solutions is enough for about 75 determinations.

5.13 D-Glucose UV test set, for quantifying glucose enzymaticly, with the hexokinase method (R-Biopharm, No. 716251[2)], according to the manufacturer, the unused kits may be stored for 1 year at 4 °C), as given in 5.13.1 to 5.13.3.

5.13.1 Buffer/substrate solution (Bottle 1)

Dissolve the content of Bottle 1 in 45 ml of freshly prepared distilled water. Store this reagent in a cool (4 °C) and dark place for not longer than for 4 weeks. Use the required amount of this solution at ambient temperature.

5.13.2 Enzyme solution (Bottle 2)

This solution is ready for use.

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1) Roche Diagnostics No. 1 202 367 is an example of a suitable product available commercially. This information is given for the convenience of users of this International Standard and does not constitute an endorsement by ISO of this product.

2) R-Biopharm AG No. 716251 is an example of a suitable product available commercially. This information is given for the convenience of users of this International Standard and does not constitute an endorsement by ISO of this products.

5.13.3 Colouring solution

Mix 22,5 ml of buffer/substrate solution (5.13.1) with 95 ml of water and 0,45 ml of enzyme solution (5.13.2) and homogenize. This amount of reagent is enough for a series of about 40 measurements. If more or less measurements are be carried out, the mixing volumes may be adapted.

Only use fresh prepared colouring solutions at ambient temperature.

6 Apparatus

Usual laboratory apparatus and, in particular, the following.

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

6.2 Centrifuge, with an acceleration of (180 ± 10) g and of 3 000 g, suitable for screw cap tubes of 12 ml (the value of g is given for the bottom of the tube).

6.3 Water bath, adjusted to (100 ± 2) °C.

6.4 Water bath, adjusted to (60 ± 1) °C.

6.5 pH-meter, calibrated, with a combined glass electrode, capable of measuring the pH to the nearest 0,01 pH unit.

6.6 Microlitre pipettes, calibrated and adjustable from 40 µl to 200 µl, 200 µl to 1 000 µl, and 1 ml to 5 ml, and a **dispenser**, adjustable from 1 ml to 5 ml.

6.7 Spectrometer, with a flow cuvette, capable of measuring at 340 nm.

6.8 Rotary shaker, speed 50 r/min, for screw-cap-closed centrifuge tubes (6.11).

6.9 Dispensor/diluter (e.g. Hook & Tucker Compudil D[3]).

6.10 Heating oven, with forced air circulation.

6.11 Centrifuge tubes, 100 mm × 16 mm, made of glass, with screw cap fitted with PTFE covered rubber seals.

6.12 Measuring flasks, of capacity 100 ml, wide-neck, equipped with a ground glass joint 14/23.

It shall be possible to measure the pH in the flask with the combined glass electrode.

6.13 Tube mixer, (e.g. Vortex mixer[3]), for the alternative method for the disintegration and solubilization of the starch, as described in 8.4.2. The following apparatus is also needed.

6.14 Shaking water bath, adjusted to (100 ± 2) °C, with a stroke of 2 cm and a shaking frequency of 150 to 200 strokes per minute. The water bath should be equipped with a holder in which the tubes fit horizontally in the water.

6.15 Centrifuge tubes, made of glass, of capacity at least 20 ml, with a screw cap fitted with PTFE covered rubber seal.

6.16 Glass pearls, 3 mm in diameter.

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3) Hook and Tucker Compudil D and Vortex are examples of suitable products available commercially. This information is given for the convenience of users of this International Standard and does not constitute an endorsement by ISO of these products.

7 Preparation of test sample

Prepare the test sample in accordance with ISO 6498.

8 Procedure

8.1 Test portion

Carry out the determination in duplicate.

Weigh approximately 200 mg of the test sample (Clause 7), to the nearest 0,1 mg, in a centrifuge tube (6.11). In a second centrifuge tube, weigh, to the nearest 0,1 mg, another 200 mg as the duplicate of the pretreated sample (Clause 7).

Repeat the procedure for each tube.

8.2 Extraction of the sugars

Add to each tube 10 ml of ethanol (5.2). Extract the solubilized sugars by rotating the suspension continuously for 10 min in the rotary shaker (6.8). Then centrifuge for 10 min in the centrifuge (6.2) with a radial acceleration of (180 ± 10) g and discard the supernatant. Repeat this extraction procedure once again.

8.3 Sample blank

From this stage in the procedure, in each analysis series a sample blank shall be analysed. Use an empty tube as a sample blank.

8.4 Disintegration of the starch

8.4.1 General

The disintegration/solubilization of starch may be carried out in two ways, i.e. according to 8.4.2 or according to 8.4.3. When the alternative procedure (see 8.4.3) is used, the larger glass reagent tubes with screw caps (6.15) shall be used.

8.4.2 Procedure I

With the dispensor under continuous vortex mixing on a vortex mixer (6.13), add 10,0 ml of aqueous DMSO solution (5.8) to the tube and keep mixing until a lump-free suspension is obtained. Close the tube with the screw cap.

NOTE Vigorous homogenization during the addition of DMSO is necessary to prevent the formation of microgel and/or lumps. Microgel and lumps will result in incorrect starch contents

— due to microgel and lumps producing an inhomogeneous sample solution, and

— the microgel and lumps will not completely be hydrolysed by AMG into glucose, so an incorrect (mostly too low) starch content will be determined.

Immediately after homogenization, attach the tube to the rotary shaker (6.8) and disintegrate the starch under continuous rotation for 30 min in an oven (6.10) set at 100 °C. Cool the tube and add, with an adjustable pipette (6.6), 1,7 ml of concentrated hydrochloric acid (5.3) and mix well. Close the tube and place it in the rotary shaker. Partly hydrolyse the sample by placing the tube under continuous rotation for 30 min in an oven (6.10) set at 60 °C.

Proceed as in 8.4.4.

8.4.3 Procedure II

Add 15 glass pearls (6.16) to the residue in the glass centrifugation tube (6.15). Add with a dispensor/diluter, under continuous vortex mixing on a Vortex mixer (6.13), 10,0 ml of aqueous DMSO solution (5.8) to the tube until a lump-free suspension is obtained. Close the tube with the screw cap.

NOTE Vigorous homogenization during the addition of DMSO is necessary to prevent the formation of microgel and/or lumps. Microgel and lumps will result in incorrect starch contents

— due to microgel and lumps producing an inhomogeneous sample solution, and

— the microgel and lumps will not completely be hydrolysed by AMG into glucose, so an incorrect (mostly too low) starch content will be determined.

Place the tube horizontally in the holder of the water bath (6.14) and shake the tube for 30 min in boiling water. Cool the tube and add, with an adjustable pipette (6.6), 1,7 ml of concentrated hydrochloric acid (5.3) and mix well. Close the tube and place the tube in a water bath adjusted to a temperature of (60 ± 1) °C. Partly hydrolyse the sample by shaking the tube for 30 min in the water bath at (60 ± 1) °C.

Proceed as in 8.4.4.

8.4.4 Adjustment of pH

Cool the tube and transfer the contents quantitatively to a 100 ml volumetric flask (6.12). Add 5,0 ml of aqueous sodium hydroxide (5.4) and 2,5 ml of sodium acetate buffer (5.7) and homogenize the solution. Measure the pH of the solution with the pH-meter (6.5). Adjust the pH, if necessary, to 4,8 ± 0,1 with dilute hydrochloric acid or sodium hydroxide. Rinse the pH electrode in the volumetric flask and dilute the solution to the mark with water.

8.5 Enzymatic conversion of the starch into glucose

Pipette (immediately after careful homogenization), with the adjustable pipette (6.6), 5,00 ml ($= V_1$ in calculation in 9.2) of the homogenized solution of dissolved and partly degraded starch (8.4.4) in a clean and dry glass tube (6.11). Add by pipette (6.6), 0,125 ml of the AMG enzymatic solution (5.12). Close the tube with a screw cap and homogenize well. Incubate the solution in a water bath set at 60 °C overnight for at least 16 h. Inactivate the AMG by placing the tube in boiling water for 15 min. Cool the tube to ambient temperature and add, by pipette, 0,125 ml of potassium hexacyanoferrate(II) solution (5.9.1) and shake for 1 min. Add ,by pipette, 0,125 ml of zinc acetate solution (5.9.2) and shake again for 1 min. The tube now contains 5,375 ml solution ($= V_2$ in calculation in 9.2). Then centrifuge for 10 min with radial acceleration of at least 3 000 g. Transfer the supernatant to a clean and dry tube.

Check for the presence of starch by adding a few millilitres of water to the centrifuge tube, boiling for 10 min, cooling, and then adding 0,2 ml of iodine solution (5.10). A blue colour indicates the presence of starch and therefore incomplete conversion. The solution for that test portion shall be discarded and the analysis for that sample restarted (see 8.1).

8.6 Enzymatic determination of the glucose content

8.6.1 Samples containing 200 g/kg to 1 000 g/kg starch

Dilute, with the dispensor/diluter (6.9), 0,5 ml of the supernatants of the hydrolysed starch solutions (8.5), the sample blank (8.3), the three standard glucose solutions (5.11), and the water blank (5.1), respectively, with 9,5 ml of water (5.1) and homogenize.

8.6.2 Samples containing 40 g/kg to 200 g/kg starch

Dilute, with the dispensor/diluter (6.9), 0,5 ml of the supernatants of the hydrolysed starch solutions (8.5), the sample blank (8.3), the three standard glucose solutions (5.11), and the water blank (5.1) respectively with 1,5 ml water (5.1) and homogenize.

If a low starch content is expected (< 200 g/kg), it is advisable to improve the sensitivity of the determination by applying another ratio of dilution for the enzymatic conversion of the starch into glucose. It is strongly recommended to use the same ratio of dilution for the sample blank. It is also recommended to prepare the standard glucose solutions at a comparable lower concentration level and to dilute these standard solutions in the same way as is done for the sample solution and the blank.

The colorimetric measurements of the blanks (8.3), the water blank (5.1), and the three glucose standards shall be carried out in duplicate. Samples containing hydrolysed starch shall be measured once.

8.6.2 Pipette, with the adjustable pipette (6.6), 0,4 ml of the diluted solution into a clean, dry centrifuge tube. Add, by pipette (6.6), 2,62 ml of colouring solution (5.13.3), and mix well. Measure the absorbance (see 6.7) of the solution at 340 nm against water.

9 Calculation and expression of results

9.1 Calibration graph

Calculate the corrected absorbance value for each of the standard glucose solutions using the equation:

$$E_{1gs} = \left(E_{0gs} - E_{0wb}\right)$$

where

E_{1gs} is the numerical value of the corrected absorbance of the standard glucose solution;

E_{0gs} is the numerical value of the absorbance of the standard glucose solution;

E_{0wb} is the numerical value of the mean of the absorbance of the water blanks.

The average corrected absorbance value of the water blanks is (by definition) equal to zero.

Using linear regression analyses, calculate the calibration graph for the corrected absorbance values against the glucose content (in grams per litre) of the undiluted standard glucose solutions.

Calculate the corrected absorbance value of each of the sample solutions using the equation:

$$E_{1s} = \left(E_{0s} - E_{0sb}\right)$$

where

E_{1s} is the numerical value of the corrected absorbance of the sample solution;

E_{0s} is the numerical value of the absorbance of the sample solution;

E_{0sb} is the numerical value of the mean of the absorbance of the sample blanks.

Using the calibration graph (9.1), calculate the glucose content (ρ_g) of the undiluted sample solutions (8.5), in grams per litre.

See Annex A.

9.2 Starch content

Calculate the starch content of the test sample using the equation:

$$w_s = \frac{\rho_g \times \frac{100}{V_1} \times V_2 \times 0{,}9}{m_0}$$

where

w_s is the numerical value of the starch content of the test sample, in grams per kilogram;

ρ_g is the numerical value of the glucose content of the sample solution, calculated according to 9.1, in grams per litre;

V_1 is the numerical value of the pipette volume of the starch solution in 8.5 (= 5,00 ml), in millilitres;

V_2 is the numerical value of the total volume after enzymatic conversion of the starch into glucose in 8.5 (= 5,375 ml), in millilitres;

m_0 is the numerical value of the mass of the test portion, in grams.

For calculating the starch content using the above equation, its is necessary that corresponding dilutions have been used for both the sample in 8.6.1 and the standard glucose solutions in 5.11.

10 Precision

10.1 Interlaboratory test

Details of an interlaboratory test on the precision of the method is summarized in Annex B. The values derived from this interlaboratory test may not be applicable to concentration ranges and matrices other than those given.

10.2 Repeatability

The absolute difference between two independent single test results, obtained using the same method on identical test material 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 be greater than the repeatability limit of

— 14 g/kg for peas, dairy compound feed and tapioca,

— 17 g/kg for piglet feed, and

— 48 g/kg for layer feed (see Annex B).

10.3 Reproducibility

The absolute difference between two single test results, obtained using the same method on identical test material in different laboratories with different operators using different equipment, will in not more than 5 % of cases be greater than the reproducibility limit of

— 25 g/kg for piglet feed,

— 34 g/kg for tapioca,

— 36 g/kg for dairy compound feed,

— 48 g/kg for layer feed, and

— 50 g/kg for peas (see Annex B).

11 Test report

The test report shall specify:

— all information necessary for the complete identification of the sample;

— the sampling method used, if known;

— the test method used, with reference to this International Standard;

— all operating details not specified in this International Standard, or regarded as optional, together with details of any incidents which may have influenced the test result(s);

— the test result obtained, or the two test results obtained if the repeatability has been checked.

Annex A
(informative)

Notes on procedure

A.1 From statistics, it is known that the reliability of a calibration curve, calculated using linear regression analysis, based on a blank and a standard solution, each measured three times, is better than a calibration curve based on a blank and five standard solutions of different concentrations, each measured once (see [5]).

A.2 In this method it is assumed that the sample matrix has no influence on the absorbance of the sample blank. It is necessary to control this every time new sample matrices are tested with this method. This can be done in the following way.

Prepare an amount of the colouring reagent according to 5.13.3, without the enzyme solution. Pipette 0,4 ml of the diluted sample solutions (prepared according to 8.6.1) to be tested, and also of the diluted sample blank, in a glass reagent tube. Pipette 2,6 ml of the colouring reagent without enzyme into these tubes.

After 30 min to 60 min, measure the absorbance of all these measuring solutions at 340 nm. The absorbance difference between the sample solutions and the sample blanks should not exceed 0,002. When for certain sample matrices this difference is larger, this method is not applicable to these sample matrices. The sample matrices studied for this International Standard, i.e. starches, animal feeding stuffs, cereals (maize, wheat and quinoa) and freeze-dried potato pulp, all meet this condition.