



International
Standard

ISO 15914-2

**Animal feeding stuffs — Enzymatic
determination of total starch
content —**

**Part 2:
Method by enzymatic determination
with a hexokinase system and
potassium hydroxide dispersion**

*Alimentation animale — Dosage enzymatique de la teneur totale
en amidon —*

*Partie 2: Méthode par dosage enzymatique avec un système
hexokinase et dispersion à l'hydroxyde de potassium*

**First edition
2024-05**

STANDARDSISO.COM : Click to view the full PDF of ISO 15914-2:2024



COPYRIGHT PROTECTED DOCUMENT

© ISO 2024

All rights reserved. Unless otherwise specified, or required in the context of its implementation, no part of this publication may be reproduced or utilized otherwise in any form or by any means, electronic or mechanical, including photocopying, or posting on the internet or an intranet, without prior written permission. Permission can be requested from either ISO at the address below or ISO's member body in the country of the requester.

ISO copyright office
CP 401 • Ch. de Blandonnet 8
CH-1214 Vernier, Geneva
Phone: +41 22 749 01 11
Email: copyright@iso.org
Website: www.iso.org

Published in Switzerland

Contents

Page

Foreword	iv
1 Scope	1
2 Normative references	1
3 Terms and definitions	1
4 Principle	1
5 Reagents	1
6 Equipment	2
7 Sampling	3
8 Preparation of the sample for testing	3
9 Operating procedure	3
9.1 Test sample	3
9.2 Washing, dispersion and hydrolysis	3
9.3 Determination of glucose	3
9.4 Control of the efficiency of the enzyme	4
10 Expression of the results	4
11 Precision	5
11.1 Interlaboratory test	5
11.2 Repeatability	5
11.3 Reproducibility	5
12 Test report	5
Annex A (normative) Check on amyloglucosidase activity	6
Annex B (informative) Result of the interlaboratory test	8
Annex C (informative) Result of the washing test	9
Bibliography	10

Foreword

ISO (the International Organization for Standardization) is a worldwide federation of national standards bodies (ISO member bodies). The work of preparing International Standards is normally carried out through ISO technical committees. Each member body interested in a subject for which a technical committee has been established has the right to be represented on that committee. International organizations, governmental and non-governmental, in liaison with ISO, also take part in the work. ISO collaborates closely with the International Electrotechnical Commission (IEC) on all matters of electrotechnical standardization.

The procedures used to develop this document and those intended for its further maintenance are described in the ISO/IEC Directives, Part 1. In particular, the different approval criteria needed for the different types of ISO document should be noted. This document was drafted in accordance with the editorial rules of the ISO/IEC Directives, Part 2 (see www.iso.org/directives).

ISO draws attention to the possibility that the implementation of this document may involve the use of (a) patent(s). ISO takes no position concerning the evidence, validity or applicability of any claimed patent rights in respect thereof. As of the date of publication of this document, ISO had not received notice of (a) patent(s) which may be required to implement this document. However, implementers are cautioned that this may not represent the latest information, which may be obtained from the patent database available at www.iso.org/patents. ISO shall not be held responsible for identifying any or all such patent rights.

Any trade name used in this document is information given for the convenience of users and does not constitute an endorsement.

For an explanation of the voluntary nature of standards, the meaning of ISO specific terms and expressions related to conformity assessment, as well as information about ISO's adherence to the World Trade Organization (WTO) principles in the Technical Barriers to Trade (TBT), see www.iso.org/iso/foreword.html.

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

A list of all parts in the ISO 15914 series can be found on the ISO website.

Any feedback or questions on this document should be directed to the user's national standards body. A complete listing of these bodies can be found at www.iso.org/members.html.

Animal feeding stuffs — Enzymatic determination of total starch content —

Part 2:

Method by enzymatic determination with a hexokinase system and potassium hydroxide dispersion

1 Scope

This document specifies an enzymatic method for determining starch in animal feeding stuffs containing starchy ingredients (cereals, tubers, etc.). The method is also applicable to beans and to the animal digestive contents because it involves the hexokinase system for the final glucose determination.

2 Normative references

The following documents are referred to in the text in such a way that some or all of their content constitutes requirements 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, *Water for analytical laboratory use — Specification and test methods*

ISO 6498, *Animal feeding stuffs — Guidelines for sample preparation*

3 Terms and definitions

No terms and definitions are listed in this document.

ISO and IEC maintain terminology databases for use in standardization at the following addresses:

- ISO Online browsing platform: available at <https://www.iso.org/obp>
- IEC Electropedia: available at <https://www.electropedia.org/>

4 Principle

Washing of the sample with a volume fraction of 40 % ethanol to eliminate the soluble sugars and soluble starch degradation products. Dispersion of the residue by means of potassium hydroxide, hydrolysis of starch into glucose units with amyloglucosidase, determination of the glucose obtained with a hexokinase system.

5 Reagents

Unless otherwise specified, use only reagents of recognized analytical grade and distilled water or water of equivalent purity.

NOTE Reagents 5.7 to 5.10 are also marketed in the form of a ready-to-use kits.

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

5.2 40 % ethanol (volume fraction) prepared as follows: Pour 400 ml of absolute ethanol into a 1 l volumetric flask and make up the volume with water.

5.3 Potassium hydroxide solution, at 1 mol/l.

5.4 Acetic acid, minimum purity of 96 %.

5.5 Amyloglucosidase of *Aspergillus niger*, glucose-free. The activity shall be checked when opening a new batch using the method described in [Annex A](#).

5.6 Aqueous solution of amyloglucosidase (5.5) with an activity of $(1\,500 \pm 100)$ units/ml and prepared extemporaneously.

A unit is defined as being a μ mole of glucose released/min/gram of enzyme.

5.7 Triethanolamine buffer solution, prepared as follows:

In a 250 ml beaker, weigh 14 g of triethanolamine hydrochloride and 0,25 g of magnesium sulfate heptahydrate, add 80 ml of water and dissolve. Then, add 5 ml of 5 N aqueous soda, homogenize and bring to pH $7,6 \pm 0,1$ using the potassium hydroxide solution ([5.3](#)). Transfer to a 100 ml volumetric flask and make up the volume with water. Agitate and keep in the refrigerator.

5.8 NADPH solution, prepared as follows:

Dissolve 60 mg of NADPH disodium salt in 6 ml of water. This solution can be kept in the refrigerator for at least four weeks.

5.9 ATP solution, prepared as follows:

In 6 ml of water, dissolve 300 mg of sodium bicarbonate and 300 mg of ATP disodium salt. This solution can be kept in the refrigerator for four weeks.

5.10 Suspension of HK/G6P-DH, prepared as follows:

Mix 1 ml of ammonium sulfate solution (3,2 mol/l), 280 U of hexokinase (EC 2.7.1.1) and 140 U of glucose-6-phosphate dehydrogenase (EC 1.1.1.49). This solution can be kept in the refrigerator for at least one year.

6 Equipment

The usual laboratory equipment and, in particular, the following shall be used.

6.1 Thermostatic bath, with magnetic or mechanical agitation, set to (60 ± 2) °C.

6.2 Centrifuge tubes, of 100 ml made of glass.

6.3 Centrifuge, for centrifuging the tubes ([6.2](#)) at about 2,000g.

6.4 Grinder, suitable for final crushing to a particle size of $\leq 0,5$ mm. The percentage of particles passing through a 0,5 mm screen shall be 95 % or more.

6.5 Spectrophotometer, ultraviolet (UV)/visible set to 340 nm or 365 nm.

6.6 pH-meter, for measurement to within 0,1 units pH.

6.7 Ultrasonic tank.

6.8 Micropipettes, for sampling volumes of 0,020 ml and 0,100 ml, verified beforehand.

6.9 Analytical balance, allowing weighing to the nearest 1 mg.

7 Sampling

Sampling is not part of the method specified in this document. A sampling method is given in ISO 6497. It is important for the laboratory to receive a truly representative sample that is not damaged or modified during transport or storage.

8 Preparation of the sample for testing

Prepare the test sample in accordance with ISO 6498.

Prior degreasing is also recommended for products with a fat content higher than 5 % (for cold degreasing, for example, fill the crucible containing the sample with acetone or petroleum ether, disperse the sample by stirring, filter and let dry).

9 Operating procedure

9.1 Test sample

To the nearest 1 mg, weigh out a test sample prepared as in [Clause 8](#), the mass of which will be calculated so that it contains (100 ± 20) mg of starch.

9.2 Washing, dispersion and hydrolysis

In a centrifuge tube ([6.2](#)) containing the test sample (see [9.1](#)) and a magnetized bar, if necessary, add 50 ml of ethanol ([5.2](#)). Agitate for 20 min at ambient temperature and then place in and activate the centrifuge ([6.3](#)) for 10 min. With a Pasteur pipette or other appropriate system, carefully extract and eliminate the liquid phase. Wash the residue twice with 25 ml of ethanol ([5.2](#)), centrifuging each time before eliminating the supernatant. Two washing operations are generally sufficient for products with a low sugar and/or fat content.

Take care to eliminate as much alcohol as possible between washings and completely evaporate it after the last washing. Washing is not essential for cereals (see [Table C.1](#)). For the other matrices, the laboratory can do an internal study in order to validate the possibility to skip the washing.

Disperse the residue in 25 ml of water and add 25 ml of potassium hydroxide ([5.3](#)). Homogenize in the ultrasonic tank ([6.7](#)) for 3 min to 5 min, then in the thermostatic bath ([6.1](#)) for 30 min. Cool and then adjust the pH to 4,6 to 4,8 with acetic acid ([5.4](#)). Then return the tube to the thermostatic bath and add 1,0 ml of enzymatic preparation ([5.6](#)). Incubate with constant agitation for 30 min.

NOTE 1 To adjust the pH, it is also possible to use an acetate buffer solution.

After cooling, transfer the total tube quantity into a 250 ml volumetric flask and make up the volume with water. Filter through filter paper. This solution is used as-is or diluted to determine the glucose and may be kept in the refrigerator for 72 h at (4 ± 2) °C. This solution may also be frozen.

Prepare a reagent blank using the conditions described above.

NOTE 2 Products containing more than 50 % amylose such as amylo maize can give questionable results.

9.3 Determination of glucose

If necessary, dilute the solution to obtain a concentration of between 100 mg and 1 g of glucose per litre. Bring the buffer solution ([5.7](#)) to ambient temperature. Measure the absorbance on the spectrophotometer

(6.5) by comparison with air or water. Using the pipetting diagram given in the [Table 1](#), analyse using micropipettes (6.8).

Table 1 — Volume of reagents to introduce

Introduce into the cuvettes	Blank ml	Test ml
Buffer solution (5.7)	1,00	1,00
NADPH (5.8)	0,10	0,10
ATP (5.9)	0,10	0,10
Test sample prepared in Clause 8	—	0,10
Water	2,00	1,90
Mix and, after about 3 min, measure the absorbance of solutions A_1 , then trigger the reaction by adding:		
HK/G6P-DH (5.10)	0,02	0,02
Mix, wait for 15 min, measure the absorbance A_2 .		

If the reaction is not completed after 15 min, continue to read the absorbance every 5 min until the absorbance increase is constant over 5 min, and extrapolate the absorbance over the additional time of suspension of HK/G6P-DH (see 5.10). For the blanks and the test, calculate the difference $\Delta A = A_2 - A_1$. Subtract the absorbance difference of the blank from that of the test.

NOTE The final volume of the test can be different from 3,22 according to the ready-to-use test used.

$$\Delta A = \Delta A_{\text{test}} - \Delta A_{\text{blank}}$$

The glucose content G , in grams per litre, in the test solution, is given by [Formula \(1\)](#):

$$G = \frac{(3,22 \times 180,16 \times \Delta A)}{(6,3 \times 1 \times 0,1 \times 1000)} = 0,92 \times \Delta A \quad (1)$$

where

3,22 is the final volume of the test;

180,16 is the molecular weight of the glucose;

6,3 is the NADPH absorption coefficient at 340 nm (or 3,5 if reading at 365 nm);

1 is the thickness of the cuvette, in centimetres;

0,1 is the test volume, in millilitres.

9.4 Control of the efficiency of the enzyme

The efficiency of the determination can be easily checked by analysing pure starch; the value obtained shall be higher than 97 % on dry matter.

10 Expression of the results

From G obtained by [Formula \(1\)](#), deduct the value of the reagent blank before carrying out the calculation described in [Formula \(2\)](#), this gives G' .

The percentage of starch expressed for the product as-is with one decimal place, is given by [Formula \(2\)](#):

$$S = \frac{G' \times 250 \times D \times 0,9}{10 \times P} \quad (2)$$

where

- S is the starch in percentage or grams per 100 g;
- G' is the concentration, in grams per litre, obtained above;
- D is the dilution factor used;
- P is the mass, in grams, of the test sample.

11 Precision

11.1 Interlaboratory test

The details of an interlaboratory test concerning the precision of the method are summarized in [Annex B](#). The values derived from this test do not necessarily apply to the concentration ranges or matrices other than those given.

11.2 Repeatability

The absolute difference between two independent individual test results, obtained with the same method, on identical test equipment, in the same laboratory, by the same operator over a short interval of time, will only exceed the repeatability limit r given in [Table B.1](#), or deduced from this table, in a maximum of 5 % of cases.

11.3 Reproducibility

The difference between the values obtained by two laboratories using this method to analyse the same laboratory sample shall not exceed the reproducibility values given in [Table B.1](#), or deduced from this table, in a maximum of 5 % of cases.

12 Test report

The test report shall include:

- all the information needed for complete identification of the sample;
- the sampling method used, if known;
- the test method used, with reference to this document, i.e. ISO 15914-2;
- all the operating details not set out in this document, i.e. ISO 15914-2, or considered to be optional, such as details regarding any incident liable to have influenced the test result(s);
- the date of the test;
- the test result(s) obtained, or if the repeatability has been confirmed, the final result obtained.

Annex A (normative)

Check on amyloglucosidase activity

A.1 General

This method enables the activity of amyloglucosidase to be measured, expressed in micromoles of glucose released per minute and per gram of enzyme (units), in the test conditions.

It shall be performed each time a new batch of enzymes is opened.

A.2 Principle

Hydrolysis of the fluid starch into glucose by means of a specified quantity of amyloglucosidase. The glucose is determined by the method described in this document (see [9.3](#)).

A.3 Reagents

Unless otherwise specified, use only reagents of recognized analytical grade and distilled water or water of equivalent purity.

A.3.1 Buffer solution pH 4,6 to 4,8, prepared as follows:

In a 100 ml volumetric flask, dissolve 8,8 g of sodium acetate trihydrate and 8,2 g (8,6 ml) of glacial acetic acid in about 50 ml of water and then make up the volume with water. Adjust the pH if necessary, to between 4,60 and 4,80. The solution may be kept in the refrigerator for one week.

A.3.2 Soluble starch, ignited residue $\leq 0,4$ %.

A.3.3 Starch solution, prepared as follows:

In a 50 ml beaker, weigh 1,6 g of starch ([A.3.2](#)) and suspend in 10 ml of water. Transfer to a 250 ml beaker containing 50 ml of boiling distilled water and boil for exactly 2 min starting at the moment large bubbles reach the surface of the liquid. Rapidly cool under cold water, add 2 ml of buffer solution ([A.3.1](#)), transfer and make up the volume in a 100 ml volumetric flask with water. Mix. This solution shall be prepared extemporaneously.

A.3.4 0,005 N sodium hydroxide solution, prepared extemporaneously.

A.4 Equipment

The usual laboratory equipment and, in particular, the following shall be used.

A.4.1 Thermostatic bath, with magnetic or mechanical agitation, set to (60 ± 2) °C.

A.4.2 Stopwatch.

A.4.3 pH-meter, allowing measurement to within 0,1 units of pH.

A.4.4 Spectrophotometer, UV/visible set to 340 nm or 365 nm.

A.4.5 Micropipettes, for sampling volumes of different volumes, verified beforehand.

A.4.6 Analytical balance, allowing weighing to the nearest 1 mg.

A.5 Operating procedure

Prepare an aqueous dilution of the enzyme such that 1 ml corresponds approximately to the release of 0,5 µmol of glucose (depending on the enzyme and its activity; for example, weigh 0,1 g to the nearest 0,1 mg (A.4.6), dissolve in 100 ml, then dilute 250 to 500 times). In two test tubes, introduce 2 ml (A.4.5) of the starch solution (A.3.3) and place in a thermostatic bath (A.4.1) for 5 min (A.4.2). Introduce the enzyme solution to be tested into each of the 2 ml tubes at an interval of 1 minute. Mix and return to the thermostatic bath (A.4.1) for exactly 15 min (A.4.2). Then add 20 ml (A.4.5) of the sodium hydroxide solution (A.3.4), agitate and leave to cool.

The glucose is determined within the hour following preparation, using the method described in 9.3 (A.4.4). That is g the result in grams per litre. Carry out a double determination.

Prepare a blank by mixing 2 ml of starch solution (A.3.3), 20 ml of sodium hydroxide solution (A.3.4) and 2 ml of the enzyme solution to be tested. Also determine the glucose on this preparation: g' (in grams per litre).

A.6 Calculation and expression of results

The activity, a , expressed in units, or micromoles of glucose per minute and per gram of enzyme, is given by Formula (A.1):

$$a = \frac{[(g - g') \times 1\,000 \times 24 \times V]}{(180,16 \times 15 \times 2 \times p)} \quad (\text{A.1})$$

where

- g is the concentration in the test tube, in grams per litre;
- g' is the concentration of the blank in the test tube, in grams per litre;
- 1 000 is the conversion factor for grams per litre to micrograms per millilitre;
- 24 is the final volume in the test tube, in millilitres;
- V is the total dilution volume of the enzyme;
- 180,16 is the molecular weight of the glucose;
- 15 is the reaction time, in minutes;
- 2 is the volume of the enzyme solution taken from the test tube;
- p is the mass of the test sample, in grams.

The result shall be considered to be the mean of the two determinations.

Annex B

(informative)

Result of the interlaboratory test

The precision of the method was established in 1995 by an interlaboratory test performed in accordance with ISO 5725-2. Twelve laboratories took part in this test. Twelve laboratories did the analyses for the rabbit compound feed and nine laboratories did the analyses for the other matrices.

[Table B.1](#) summarizes the statistical results of the test.

Table B.1 — Statistical results

Variable	Sample				
	1 Corn starch	2 Manioc	3 Corn	4 Turkey feed	5 Rabbit feed
Participating laboratories	9	9	9	9	12
Number of laboratories selected after elimination of aberrant values	9	9	9	9	12
Mean starch content, g/100 g	85,6	69,3	61,8	33,9	9,4
Repeatability standard deviation (s_r), g/100 g	0,4	0,5	0,2	0,5	0,1
Repeatability variation coefficient, %	0,5	0,7	0,4	1,6	1,5
Repeatability limit (r) [$r = 2,8 \times s_r$], g/100 g	1,2	1,3	0,7	1,5	0,4
Reproducibility standard deviation (s_R), g/100 g	1,4	1,1	2,3	1,8	0,4
Reproducibility variation coefficient, %	1,6	1,7	3,7	5,3	4,5
Reproducibility limit (R) [$R = 2,8 \times s_R$], g/100 g	4,0	3,2	6,5	5,1	1,2