INTERNATIONAL STANDARD

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Modified starch — Determination of acetyl content — Enzymatic method

Amidon modifié — Dosage de l'acétyle — Méthode enzymatique

Reference number
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Foreword

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International Standard ISO 11213 was prepared by Technical Committee ISO/TC 93, *Starch (including derivatives and by-products)*.

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

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Modified starch — Determination of acetyl content — Enzymatic method

1 Scope

This International Standard specifies an enzymatic method for the determination of the acetyl content of modified starch, both granular and soluble in cold water. Total and free acetyl contents are determined and the bound acetyl content is calculated.

The method is suitable for determining acetyl contents up to 2 % (*m/m*).

2 Normative references

The following standards contain provisions which, through reference in this text, constitute provisions of this International Standard. At the time of publication, the editions indicated were 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 editions of the standards indicated below. Members of IEC and ISO maintain registers of currently valid International Standards.

ISO 1666:1973, *Starch — Determination of moisture content — Oven-drying methods.*

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

3 Principle

The total acetyl content is determined by heating the sample with dilute hydrochloric acid which hydrolyses the acetyl fraction and solubilizes the starch. In the presence of the enzyme acetyl-CoA synthetase (ACS), acetate is converted with adenosine-5-triphosphate (ATP) and coenzyme A (CoA) to acetyl-Co-A. The latter then reacts with oxaloacetate to form citrate in the presence of citrate synthase (CS).

The oxaloacetate required for the reaction is formed from malate and nicotinamide adenine dinucleotide (NAD) in the presence of malate-dehydrogenase (MDH). In this reaction, the NAD is reduced to NADH and the formation of NADH can be determined by measuring the increase in absorbance at a specified wavelength. (See reference [1] in annex C.)

The free acetyl content is determined by making a suspension of the modified starch in water, filtering, and determining the acetyl content of the filtrate as already described. The bound acetyl content is calculated by subtracting the free acetyl content from total acetyl content.

4 Reagents and materials

The reagents used shall be of recognized analytical grade, unless otherwise specified. The water used shall comply with the specifications of ISO 3696, grade 2. The enzymes used shall be of a quality equivalent to the relevant enzymes of Boehringer Mannheim[1].

NOTE 1 Suitable test kits which are commercially available can be used.

4.1 Hydrochloric acid, 1 mol/l solution.

4.2 Sodium hydroxide, 5 mol/l solution.

4.3 Buffer solution.

In about 70 ml of water, dissolve the following reagents:

1) This information is given for the convenience of users of this International Standard and does not constitute an endorsement by ISO of the products named.

7,5 g of triethanolamine;

420 mg of L-malic acid;

210 mg of magnesium chloride hexahydrate ($MgCl_2.6H_2O$).

Add as much potassium hydroxide 5 mol/l solution as necessary in order to obtain a pH of 8,4. The volume required is about 8 ml.

The solution is stable for 1 year when stored at + 4 °C.

4.4 ATP-CoA-NAD solution.

In 20 ml of water, dissolve the following reagents:

500 mg of crystallized disodium salt trihydrate of adenosine-5'-triphosphate [ATP-$Na_2H_2.3H_2O$; 98 % (*m/m*)];

500 mg of anhydrous sodium hydrogen carbonate;

50 mg of lyophilized trilithium salt of coenzyme A [about 85 % (*m/m*) CoA];

250 mg of lyophilized free acid monohydrate of nicotinamide adenine dinucleotide [β-NAD.H_2O > 98 % (*m/m*)].

The solution is stable for 1 week when stored at + 4 °C.

4.5 MDH-CS suspension.

Disperse about 1 100 U (international units) of malate-dehydrogenase (MDH from pig heart; EC 1.1.1.37) and about 270 U of citrate synthase (CS from pig heart; EC 4.1.37) in 0,4 ml of ammonium sulfate solution, $c[(NH_4)_2SO_4]$ = 3,2 mol/l.

The solution is stable for 1 year when stored at + 4 °C.

NOTE 2 One international unit (1 U) catalyses 1 µmol/min at 25 °C from the relevant substrate.

4.6 ACS solution.

Dissolve 20 mg of lyophilizate containing 5 mg of acetyl-coenzyme A synthetase (ACS from yeast; EC 6.2.1.1; ≈ 16 U) in 0,4 ml of water.

The solution is stable for 5 d when stored at + 4 °C.

5 Apparatus

Usual laboratory apparatus and in particular the following.

5.1 Conical flasks, of capacity 250 ml, equipped with screw caps.

5.2 Boiling water bath, equipped with a shaker.

5.3 Volumetric flasks, of capacity 200 ml.

5.4 Micropipettes or **syringes**.

5.5 Molecular absorption spectrometer, suitable for operation at 340 nm.

5.6 Cuvettes, of quartz glass or other materials transparent at 340 nm, with a thickness of 10 mm ± 0,1 mm.

5.7 Sieve, with an aperture of 800 µm.

5.8 Blade mill.

5.9 Water bath, capable of being thermostatically controlled between 20 °C and 25 °C.

6 Preparation of the sample

Sieve through a 800 µm sieve (5.7). If material does not pass through the sieve, grind the sample with a blade mill (5.8) so that it will completely pass through the 800 µm sieve. Homogenize the sample.

7 Procedure

7.1 Hydrolysis of acetyl groups

7.1.1 Dispersion of granular starch

Weigh, to the nearest 1 mg, approximately 1 g of the prepared sample and place it in a conical flask (5.1).

Add 50 ml of hydrochloric acid (4.1) while agitating to ensure good dispersion. Continue as described in 7.1.3.

7.1.2 Dispersion of pregelatinized starch

Add 50 ml of hydrochloric acid (4.1) to a conical flask (5.1). Introduce a magnetic stirrer and start agitation. Slowly and carefully add about 1 g of the prepared sample. Ensure a good, lump-free dispersion. Determine the mass of the test portion by weighing by difference to the nearest 1 mg.

7.1.3 Hydrolysis and filtration

Seal the conical flask tightly with the screw cap and place it in the boiling water bath (5.2) with the shaker operating for 30 min.

Remove the flask and cool to about 20 °C ± 5 °C by immersing it in an ice bath. Open the flask when its content is fully cooled, add 10 ml of sodium hydroxide solution (4.2), mix, and wash the contents quantitatively into a 200 ml volumetric flask (5.3). Place the volumetric flask in the water bath (5.9) for temperature equilibration between 20 °C and 25 °C. Control the temperature and make up to the mark with distilled water. Filter through a suitable filter paper. Reject the first 20 ml to 30 ml of filtrate and directly use the remaining solution as the test solution in the enzymatic determination, as described in 7.4.

7.2 Free acetate

7.2.1 Dispersion of granular starch

Disperse 10 g of the prepared sample, while stirring, in 100 ml of distilled water in a conical flask (5.1).

Continue as described in 7.2.3.

7.2.2 Dispersion of pregelatinized starch

Add about 100 ml of distilled water to a conical flask (5.1). Introduce a magnetic stirrer and start agitation. Slowly and carefully add about 2 g of the prepared sample. Ensure a good, lump-free dispersion. Determine the mass of the test portion by weighing by difference to the nearest 1 mg.

7.2.3 Dissolution and filtration

Seal the conical flask and agitate for 30 min.

Transfer the contents of the conical flask quantitatively to a 200 ml volumetric flask (5.3). Place the volumetric flask in the water bath (5.9) for temperature equilibration between 20 °C and 25 °C. Control the temperature and make up to the mark with distilled water. Filter through a suitable filter paper. Reject the first 20 ml to 30 ml of filtrate and directly use the remaining solution as the test solution in the enzymatic determination, as described in 7.4.

7.3 Check test

To check the method, the assay can be performed on a reference material such as pure anhydrous sodium acetate [acetyl content = 52,4 % (*m/m*)]. For this, weigh to the nearest 0,1 mg, about 100 mg of anhydrous sodium acetate. Then transfer to a 1 000 ml volumetric flask. Place the volumetric flask in the water bath (5.9) for temperature equilibration between 20 °C and 25 °C. Control the temperature and make up to the mark with distilled water. Continue as described in 7.4.

7.4 Enzymatic determination of acetic acid

Carry out the enzymatic determination of acetic acid according to the following analytical arrangement and conditions:

— wavelength: 340 nm;

— temperature: 20 °C to 25 °C.

Read the absorbances against a cuvette (5.6) filled with water.

NOTE 3 Measurements can also be made at the following wavelengths with the corresponding molar absorption coefficients ($\varkappa$) used in the calculations:

— Hg, 365 nm: $\varkappa = 3{,}4\ \text{l·mmol}^{-1}\text{·cm}^{-1}$;

— Hg, 334 nm: $\varkappa = 6{,}18\ \text{l·mmol}^{-1}\text{·cm}^{-1}$.

Pipette into cuvettes (5.6) the volumes of reagents indicated in the analytical arrangement in table 1.

8 Expression of results

8.1 Absorbance difference

Calculate the absorbance difference using the equation:

$$\Delta A = \left[(A_2 - A_0)_s - \frac{(A_1 - A_0)_s^2}{(A_2 - A_0)_s} \right] - \left[(A_2 - A_0)_b - \frac{(A_1 - A_0)_b^2}{(A_2 - A_0)_b} \right]$$

where

ΔA is the numerical value of the absorbance difference;

A_0, A_1 and A_2 are the numerical values of absorbances measured according to the analytical arrangement of table 1;

s is an index designating the solution with sample;

b is an index designating the blank.

Table 1 — Analytical arrangement for enzymatic determination of acetic acid

Reagent and action	Blank	Solution with sample
Buffer solution (4.3)	1,00 ml	1,00 ml
ATP-CoA-NAD solution (4.4)	0,20 ml	0,20 ml
Double-distilled water	2,00 ml	1,50 ml
Test solution	—	0,50 ml
Mix the contents of each cuvette and read the absorbance (A_0). To each cuvette add		
MDH-CS suspension (4.5)	0,01 ml	0,01 ml
Mix the contents of each cuvette and read the absorbance (A_1) after about 3 min. Start the reaction by adding to each cuvette		
ACS solution (4.6)	0,02 ml	0,02 ml
Mix the contents of each cuvette, wait until the reaction has stopped (about 10 min to 15 min) and read the absorbance (A_2). If the reaction has not stopped after about 15 min, continue to read the absorbance at 2 min intervals until the absorbance increases constantly for 2 min.		
NOTE — The volume of solution with sample can be changed to accommodate very high or very low acetate levels; the final total volume must always be 3,23 ml.		

8.2 Total acetyl content

Calculate the total acetyl content using the equation

$$w_a = \frac{5{,}56 \times \Delta A}{\varkappa \times m_1} \times \frac{100}{100 - w_m}$$

where

w_a is the numerical value of the total acetyl content, in percentage by mass, of the test sample;

ΔA is the numerical value of the absorbance difference calculated according to 8.1;

$\varkappa$ is the numerical value of the molar absorption coefficient of NADH at 340 nm, in litres per millimole centimetre $(\varkappa = 6{,}30 \text{ l·mmol}^{-1}\text{·cm}^{-1})$;

m_1 is the numerical value of the mass, in grams, of the test portion (see 7.1.1 or 7.1.2);

w_m is the numerical value of the moisture content, in percentage by mass, of the sample.

If an aliquot of solution with sample other than the 0,50 ml specified in table 1 is taken, the equation has to be adjusted accordingly. When the check test (7.3) is effected, a dilution factor of 5 has to be included because the test solution is made up to 1 000 ml and not 200 ml as in the procedure for the test sample (see 7.1 and 7.2).

NOTE 4 A full explanation of the derivation of the equation is given in annex A.

8.3 Free acetyl content

Calculate the free acetyl content using the equation

$$w_f = \frac{5{,}56 \times \Delta A}{\varkappa \times m_2} \times \frac{100}{100 - w_m}$$

where

w_f is the numerical value of the free acetyl content, in percentage by mass, of the test sample;

ΔA, $\varkappa$ and w_m have the same meanings as in 8.2;

m_2 is the numerical value of the mass, in grams, of the test portion (see 7.2.1 or 7.2.2).

8.4 Bound acetyl content

Calculate the bound acetyl content using the equation

$$w_{ba} = w_a - w_f$$

where

w_{ba} is the numerical value of the bound acetyl content, in percentage by mass, of the test sample;

w_a is the numerical value of the total acetyl content, in percentage by mass, of the test sample;

w_f is the numerical value of the free acetyl content, in percentage by mass, of the test sample.

9 Precision

The precision of the method was established by an interlaboratory test organized by ISO/TC 93/WG 3, *Chemical functions*, in 1990 and carried out in accordance with ISO 5725 (reference [2] in annex C). In this test 11 laboratories participated: samples of modified maize starch, modified potato starch and modified wheat starch were investigated. See annex B for a summary of the statistical results of this test. The probability level is 95 % when the repeatability and reproducibility limits are obtained.

9.1 Repeatability

The absolute difference between two independent 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, should not be greater than 5 % of the higher of the two results.

9.2 Reproducibility

The absolute difference between two independent test results, obtained using the same method on identical test material in different laboratories with different operators using different equipment, should not be greater than 8 % of the higher of the two results.

10 Test report

The test report shall specify

— a reference to this International Standard;

— the sampling method used;

— the method used;

— the test result(s) obtained;

— if the repeatability has been checked, the final quoted result obtained.

It shall also mention 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 report shall include all information necessary for the complete identification of the sample.

Annex A
(informative)

Derivation of equation for calculation of acetyl content

The total acetyl concentration in the test solution is calculated using the equation

$$\rho_{at} = \frac{M_r \times V_2 \times \Delta A}{1\ 000 \times V_1 \times d \times \varkappa}$$

where

ρ_{at} is the numerical value of the total acetyl concentration, in grams per litre, in the test solution;

M_r is the relative molecular mass of the acetyl function ($M_r = 43$);

V_1 is the numerical value of the volume, in millilitres, of the test solution ($V_1 = 0{,}50$ ml);

V_2 is the numerical value of the final volume, in millilitres, of the solution with sample ($V_2 = 3{,}23$);

ΔA is the numerical value of the absorbance difference calculated according to 8.1;

d is the numerical value of the thickness, in centimetres, of the cuvette ($d = 1$ cm);

$\varkappa$ is the numerical value of the molar absorption coefficient of NADH at 340 nm, in litres per millimole centimetre $\left(\varkappa = 6{,}30\ \mathrm{l \cdot mmol^{-1} \cdot cm^{-1}}\right)$.

The total acetyl content of the humid test sample is calculated using the equation

$$w_{ah} = \frac{\rho_{at} \times V_3}{10 \times m_1}$$

where

w_{ah} is the numerical value of the total acetyl content, in percentage by mass, of the humid test sample;

ρ_{at} is the numerical value of the total acetyl concentration, in grams per litre, in the test solution;

V_3 is the numerical value of the volume, in millilitres, of the test solution before filtration ($V_3 = 200$ ml);

m_1 is the numerical value of the mass, in grams, of the test portion (see 7.1.1 or 7.1.2).

Thus

$$w_{ah} = \frac{M_r \times V_2 \times \Delta A}{1\ 000 \times V_1 \times d \times \varkappa} \times \frac{V_3}{10 \times m_1}$$

$$= \frac{43 \times 3{,}23 \times 200 \times \Delta A}{1\ 000 \times 0{,}50 \times 1 \times 10 \times \varkappa \times m_1}$$

$$= \frac{5{,}56 \times \Delta A}{\varkappa \times m_1}$$

The total acetyl content of the test sample, based on dry matter, is calculated using the equation

$$w_a = w_{ah} \frac{100}{100 - w_m} = \frac{5{,}56 \times \Delta A}{\varkappa \times m_1} \times \frac{100}{100 - w_m}$$

where w_m is the numerical value of the moisture content, in percentage by mass, of the sample.

Annex B
(informative)

Statistical results of the interlaboratory test

Parameter	Sample[1)]				
	WL	ML	MH	PL	PH
Number of laboratories retained after eliminating outliers	10	10	10	11	11
Number of outliers (laboratories)	1	1	1	0	0
Number of accepted results	20	20	20	22	22
Mean acetyl content [% (*m/m*)]	1,00	0,70	1,19	0,61	1,92
Repeatability standard deviation, s_r [% (*m/m*)]	0,006	0,013	0,013	0,007	0,050
Repeatability variation coefficient, VC_r, %	0,6	1,9	1,08	1,20	2,61
Repeatability limit, $r = 2{,}8 \times s_r$ [% (*m/m*)]	0,017	0,037	0,036	0,021	0,142
Reproducibility standard deviation, s_R [% (*m/m*)]	0,009	0,015	0,020	0,024	0,088
Reproducibility variation coefficient, VC_R, %	0,95	2,16	1,69	3,97	4,60
Reproducibility limit, $R = 2{,}8 \times s_R$ [% (*m/m*)]	0,027	0,042	0,057	0,069	0,250

1) H high content
L low content
M modified maize starch
P modified potato starch
W modified wheat starch