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**Modified starch — Determination of acetyl
content — Enzymatic method**

Amidon modifié — Dosage de l'acétyle — Méthode enzymatique

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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)*.

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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 Mannheim¹⁾.

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 ($\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$).

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 [$\text{ATP-Na}_2\text{H}_2\text{.3H}_2\text{O}$; 98 % (*m/m*)];

500 mg of anhydrous sodium hydrogen carbonate;

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

250 mg of lyophilized free acid monohydrate of nicotinamide adenine dinucleotide [$\beta\text{-NAD.H}_2\text{O}$ > 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, $c[(\text{NH}_4)_2\text{SO}_4] = 3,2 \text{ mol/l}$.

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

NOTE 2 One international unit (1 U) catalyses 1 $\mu\text{mol/min}$ 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; $\approx 16 \text{ U}$) 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 $10 \text{ mm} \pm 0,1 \text{ mm}$.

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 $20\text{ °C} \pm 5\text{ °C}$ 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 (κ) used in the calculations:

- Hg, 365 nm: $\kappa = 3,4\text{ l}\cdot\text{mmol}^{-1}\cdot\text{cm}^{-1}$;
- Hg, 334 nm: $\kappa = 6,18\text{ l}\cdot\text{mmol}^{-1}\cdot\text{cm}^{-1}$.

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.