# INTERNATIONAL STANDARD

**ISO 8451**

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# Tobacco — Determination of starch content — Enzymatic method



*Tabac — Détermination de la teneur en amidon — Méthode enzymatique*



INTERNATIONAL ISO

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## 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 8451 was prepared by Technical Committee ISO/TC 126, *Tobacco and tobacco products*, Sub-Committee SC 2, *Leaf tobacco*.



Annex A of this International Standard is for information only.



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# Tobacco — Determination of starch content — Enzymatic method

## 1 Scope

This International Standard specifies a method for the determination of natural starch in tobacco and tobacco products. The method is applicable to all types of tobacco and tobacco products.

NOTES

1 Modified kinds of starch (phosphorylized or oxidized) do not react.

2 If this method is used for tobaccos or tobacco products that have been cased with sugars, determine glucose before hydrolysis and then correct the glucose determination after analysis for the proper determination of starch content.



## 2 Definition

For the purposes of this International Standard, the following definition applies.

**2.1 starch content of tobacco:** The content of the substances determined by the procedure specified in this International Standard and expressed as a percentage of starch ($m/m$).

## 3 Reactions

In the presence of the enzyme amyloglucosidase (AGS), starch is hydrolyzed to glucose at pH 4,6 [equation (1)].

$$\text{Starch} + (n-1)\,(H_2O) \xrightleftharpoons{\text{AGS}} n\ \text{glucose} \qquad \ldots (1)$$

The glucose formed is determined with hexokinase (HK) and glucose-6-phosphate dehydrogenase (G6P-DH) at pH 7,6. Glucose is phosphorylated to glucose-6-phosphate (G-6-P) by adenosine-5′-triphosphate (ATP) [equation (2)] in the presence of hexokinase.

$$\text{Glucose} + \text{ATP} \xrightleftharpoons{\text{HK}} \text{G-6-P} + \text{ADP} \qquad \ldots (2)$$

In the presence of G6P-DH, glucose-6-phosphate is oxidized by nicotinamide adenine dinucleotide phosphate (NADP) to gluconate-6-phosphate (G-6-P) with formation of the reduced form of NADP (NADPH) [equation (3)].

$$\text{G-6-P} + \text{NADP}^+ \xrightleftharpoons{\text{G6P-DH}} \text{gluconate-6-phosphate} + \text{NADPH} + \text{H}^+ \qquad \ldots (3)$$

The amount of NADPH formed in the above reaction is stoichiometric with the amount of glucose. NADPH is determined by means of its absorbance at 334 nm, 340 nm or 365 nm.

## 4 Reagents and preparation of the solutions

### 4.1 Reagents

Use only reagents of recognized analytical grade and freshly redistilled water in glassware.

**4.1.1 Citric acid monohydrate**, $C_6H_8O_7.H_2O$.

**4.1.2 Trisodium citrate dihydrate**, $C_6H_5Na_3O_7.2H_2O$.

**4.1.3 Amyloglucosidase, AGS**, about 6 units/mg lyophilisate.

**4.1.4 Triethanolamine hydrochloride**, $C_6H_{15}NO_3.HCl$.

**4.1.5 Magnesium sulfate heptahydrate**, $MgSO_4.7H_2O$.

**4.1.6 Sodium hydroxide solution**, $c(NaOH) = 5$ mol/l.

**4.1.7 Nicotinamide adenine dinucleotide phosphate-disodium salt**, $NADP\text{-}Na_2$.

**4.1.8 Adenosine-5′ triphosphate-disodium salt**, $ATP\text{-}Na_2H_2$.

**4.1.9 Sodium hydrogen carbonate**, $NaHCO_3$.

**4.1.10 Hexokinase/glucose-6-phosphate dehydrogenase**, HK/G6P-DH.

**4.1.11 Dimethyl sulfoxide**, $CH_3SOCH_3$.

**4.1.12 Hydrochloric acid**, $c(HCl) = 8$ mol/l.

### 4.2 Preparation of the solutions

#### 4.2.1 Citrate buffer, pH 4,6

Dissolve 88 mg citric acid monohydrate (4.1.1) and 170 mg trisodium citrate dihydrate (4.1.2) in water and dilute to 20 ml. Check the pH value of 4,6 by glass electrode.

The buffer is stable for at least 1 year at + 4 °C.

Bring the buffer solution to 20 °C to 25 °C before use.

#### 4.2.2 Amyloglucosidase (AGS) solution

Dissolve 29 mg lyophilized AGS (4.1.3) in 1,2 ml citrate buffer (4.2.1).

The solution is stable for at least 6 months at + 4 °C.

#### 4.2.3 Triethanolamine buffer, pH 7,6

Dissolve 14 g triethanolamine hydrochloride (4.1.4) and 0,25 g magnesium sulfate heptahydrate (4.1.5) in approximately 80 ml water, adjust pH to 7,6 with approximately 5 ml sodium hydroxide solution (4.1.6) and dilute to 100 ml with water.

The buffer is stable for at least 4 weeks at + 4 °C.

Bring the buffer solution to 20 °C to 25 °C before use.

#### 4.2.4 Nicotinamide adenine dinucleotide phosphate (NADP) solution

Dissolve 60 mg nicotinamide adenine dinucleotide phosphate-disodium salt (4.1.7) in 6 ml water.

The solution is stable for at least 4 weeks at + 4 °C.

#### 4.2.5 Adenosine-5′-triphosphate (ATP) solution

Dissolve 300 mg adenosine-5′ triphosphate-disodium salt (4.1.8) and 300 mg sodium hydrogen carbonate (4.1.9) in 6 ml water.

The solution is stable for at least 4 weeks at + 4 °C.

#### 4.2.6 Hexokinase/glucose-6-phosphate dehydrogenase

HK/G6P-DH (2 mg HK/ml suspension in 3,2 mol/l ammonium sulfate solution; 1 mg G6P-DH/ml suspension in 3,2 mol/l ammonium sulfate solution).

Use the suspension undiluted

The suspension is stable for at least 1 year at + 4 °C.

## 5 Apparatus

Usual laboratory equipment, and in particular



**5.1 Spectrometer**, suitable for making measurements at 340 nm.

NOTE 3 Spectral line filter photometers suitable for making measurements at 334 nm and 365 nm (mercury lamps) may also be used.



**5.2 Laboratory mill.**.

**5.3 Wire sieve**, 0,3 mm sieve aperture.

**5.4 Glass cuvette**, 10 mm optical path length.

**5.5 Narrow neck volumetric flask**, of capacity 100 ml.

**5.6 Water bath**, capable of being controlled at 55 °C to 60 °C.

**5.7 pH-meter.**.

**5.8 Filter paper**, fast filtering grade.

**5.9 Micropipettes.**.

**5.10 Drying oven**, heating up to 60 °C.

NOTE 4 All pipettes and cuvettes should be cleaned before use by using chromic acid solution or special detergents.

## 6 Procedure

### 6.1 Preparation of the test sample

Dry the test sample in the drying oven (5.10) **at a temperature not higher than 60 °C**. Then grind the test sample. The complete sample shall pass through the wire sieve (5.3).

### 6.2 Test portion

Weigh, to the nearest 0,001 g, 0,5 g to 1 g of the well-mixed ground test sample in a 100 ml volumetric flask (5.5). Carry out the analysis in duplicate.

### 6.3 Extraction

Add 20 ml dimethyl sulfoxide (4.1.11) and 5 ml hydrochloric acid (4.1.12) and incubate for 2 h **at 60 °C** in a water bath, shaking periodically. Cool to room temperature and add approximately 50 ml water. Adjust the solution to pH 4 to 5 using sodium hydroxide solution (4.1.6) with vigorous shaking. Dilute to the mark with water and filter the solution. Discard the first 10 ml of the filtrate and use 0,1 ml (maximum 0,2 ml) for the assay immediately.

### 6.4 Determination

The procedure for the determination is as follows:

| Pipette into cuvette | Sample blank | Sample |
|---|---|---|
| Citrate buffer (4.2.1) | — | 0,2 ml |
| Sample solution | 0,1 ml | 0,1 ml |
| Amyloglucosidase (4.2.2) | — | 0,02 ml |

Mix and incubate for 15 min **at 55 °C to 60 °C** in the water bath (5.6); stopper the cuvette with the lid. Place the cuvette into the spectrometer (5.1).

NOTE 5 The contents of the cuvette are mixed well by using a small plastic or glass rod during the incubations.

Add

| | | |
|---|---|---|
| Triethanolamine buffer (4.2.3) | 1 ml | 1 ml |
| NADP (4.2.4) | 0,1 ml | 0,1 ml |
| ATP (4.2.5) | 0,1 ml | 0,1 ml |
| Redistilled water | 1,72 ml | 1,5 ml |

Mix. After approximately 3 min read the absorbance of the solutions ($A_1$). Start the reaction by the addition of

| | | |
|---|---|---|
| HK/G6P-DH (4.2.6) | 0,02 ml | 0,02 ml |

Mix. After completion of the reaction (about 10 min to 15 min) read the absorbance of the solutions ($A_2$). If the reaction has not stopped after 15 min read absorbance at 5 min intervals until the reading is constant for 5 min.

In case of constant increase of the absorbances of $A_2$, extrapolate the absorbance to the time of addition of the HK/G6P-DH suspension (see annex A).

Calculate the absorbance differences as follows:

$$\Delta A_{\text{sample blank}} = A_{2\ \text{sample blank}} - A_{1\ \text{sample blank}}$$

$$\Delta A_{\text{sample}} = A_{2\ \text{sample}} - A_{1\ \text{sample}}$$

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



## 7 Expression of results

### 7.1 Calculation

In accordance with the general formula, the equation for concentration is

$$c = \frac{V_1 \cdot M_S \cdot F}{\epsilon \cdot d \cdot V_2 \cdot 1\,000} \Delta A \ \text{(g/l)}$$

where

$V_1$ is the final volume, in millilitres;

$V_2$ is the sample volume, in millilitres;

$M_S$ is the relative molecular mass of the substance to be assayed (for starch: $M_{\text{glucose}} - M_{\text{water}} = 162{,}1$);

$F$ is the dilution factor (if the sample was diluted during the preparation);

$d$ is the optical path length, in centimetres;

$\epsilon$ is the absorption coefficient of NADPH at

340 nm = 6,3 ($l \cdot mmol^{-1} \cdot cm^{-1}$)

Hg 365 nm = 3,5 ($l \cdot mmol^{-1} \cdot cm^{-1}$)

Hg 334 nm = 6,18 ($l \cdot mmol^{-1} \cdot cm^{-1}$)

It follows for starch

$$c = \frac{3{,}04 \cdot 162{,}1 \cdot 1}{\epsilon \cdot 1 \cdot 0{,}1 \cdot 1\,000} \cdot \Delta A = 4{,}928$$

$$\frac{\Delta A}{\epsilon}$$ (g starch/litre sample solution)

$$\% \text{ starch} = \frac{100 \cdot c \cdot 10}{m \cdot (100 - W)}$$

where

$c$ is the concentration, in grams per litre, of starch in the sample solution;

$m$ is the mass, in grams, of the test portion (6.2);

$W$ is the water content of the prepared tobacco sample (6.1), in per cent.

### 7.2 Repeatability

If the difference between the results of two determinations, carried out on the same day by the same analyst using the same apparatus and the same sample, exceed 0,2 % ($m/m$) of starch, then two further determinations shall be carried out.

### 7.3 Reproducibility

The difference between two single values obtained by two determinations in different laboratories, using portions of the same sample shall not exceed 0,2 % ($m/m$) of starch.

## 8 Test report

The test report shall show the method used and the results obtained. It shall also mention any operating details not specified in this International Standard, or regarded as optional, together with details of any incidents likely to have influenced the results.

The test report shall include all information necessary for the complete identification of the sample.

# Annex A
(informative)

## "Normal" and "slow-speed" reactions

See figure A.1.

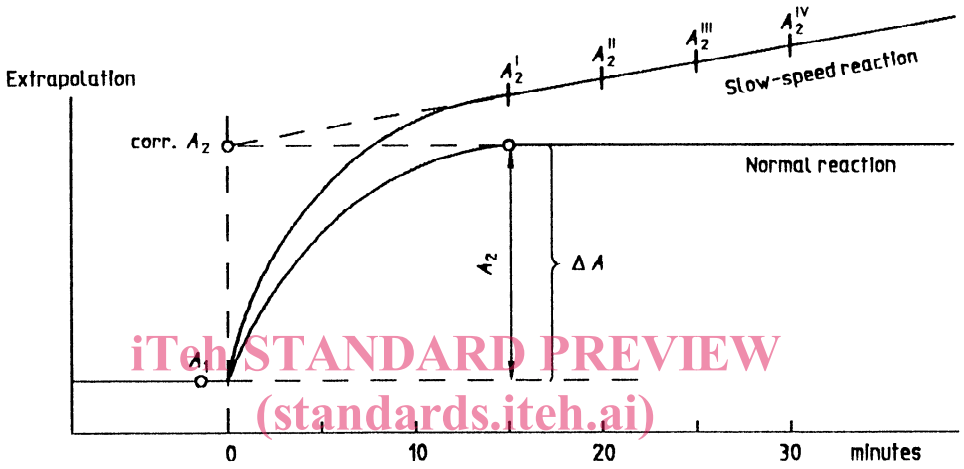




**Figure A.1**



"Normal" reaction

$$\Delta A = A_2 - A_1$$

"Slow-speed" reaction

$A_1$ is measured, after 15 min $A_2$ is measured. The values measured after a further 5 min each are $A_2^{II}$, $A_2^{III}$, $A_2^{IV}$, ... .

A slow-speed reaction exists if the differences of absorption from $A_2^{I}$ to $A_2^{II}$, or from $A_2^{II}$ to $A_2^{III}$ respectively, etc. are constant.

Graphically the corrected $A_2$ can be found if the straight line $A_2^{I} - A_2^{II}$ is extended more than $A_2'$ up to the time of the addition of the initial enzyme (0 min, corr. $A_2$).

Mathematically (by extrapolation) the correct $A_2$ can be obtained in accordance with

$$\text{corr. } A_2 = \underset{(=15\text{ min})}{A_2'} - 3 \times \underset{(=5\text{ min})}{(A_2'' - A_2^{I})}$$

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