International Standard 5542

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Milk — Determination of protein content — Amido black dye-binding method (Routine method)

Lait — Détermination de la teneur en protéines — Méthode au noir amido (Méthode pratique)

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Foreword

ISO (the International Organization for Standardization) is a worldwide federation of national standards bodies (ISO member bodies). The work of developing International Standards is carried out through ISO technical committees. Every member body interested in a subject for which a technical committee has been authorized 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.

Draft International Standards adopted by the technical committees are circulated to the member bodies for approval before their acceptance as International Standards by the ISO Council.

International Standard ISO 5542 was developed by Technical Committee ISO/TC 34, *Agricultural food products*, and was circulated to the member bodies in April 1983.

It has been approved by the member bodies of the following countries :

Australia	Iran	South Africa, Rep. of
Austria	Iraq	Spain
Belgium	Korea, Dem. P. Rep. of	Sri Lanka
Canada	Malaysia	Tanzania
Cuba	Netherlands	Thailand
Czechoslovakia	New Zealand	Turkey
Egypt, Arab Rep. of	Philippines	United Kingdom
France	Poland	USSR
Germany, F. R.	Portugal	Yugoslavia
Hungary	Romania	

No member body expressed disapproval of the document.

NOTE — The method specified in this International Standard has been developed jointly with the International Dairy Federation (IDF) and the Association of Official Analytical Chemists (AOAC) and will also be published by these organizations.

Milk — Determination of protein content — Amido black dye-binding method (Routine method)

1 Scope and field of application

1.1 This International Standard describes the amido black dye-binding method, used as a routine method, for the determination of the protein content of milk.

As the composition of the amido black dyestuff is variable, the method described is empirical and depends upon constant reference to the protein content derived from determination of the nitrogen content of milk by the Kjeldahl reference method (for example, as described in IDF Standard 20).

1.2 The method is applicable to raw or thermally or mechanically processed (for example pasteurized, sterilized, homogenized, reconstituted) whole milk, partially skimmed milk and skimmed milk, provided that the samples are in good condition. The method is also applicable, in some cases, to preserved samples (see 10.1).

The method allows a rapid and simple determination of the protein content of milk to be made and is suitable for a single determination or for determinations on small numbers of samples and for series of multiple determinations. For determinations in series, special apparatus (i.e. multiple pipetting apparatus, centrifuges for racks) is required (see 6.4 and 6.7), and frequent checking of control samples for correction of "drift" is required (see 8.6.2). In view of the time-consuming method of calibration, laboratories which make only a few determinations on certain types of samples often have recourse to central laboratories for dye solutions and control samples.

NOTE — According to the origin of the sample and the reference method used, the method described in this International Standard may be used not only for the usual determination of the protein content of milk (i.e. total nitrogen × 6,38), but also for the determination of the "true protein" content, or with modifications, the casein or whey protein content of cow's milk and milk from other species (goat, sheep, etc.).

2 References

ISO 707, *Milk and milk products — Methods of sampling.*

IDF Standard 20 : *Determination of the nitrogen content of milk by the Kjeldahl method.*

3 Definition

protein content : A conventional value obtained by multiplying the nitrogen content, expressed as a percentage by mass, determined in accordance with the Kjeldahl reference method (for example, as described in IDF Standard 20), by an appropriate factor.

NOTE — Care should be taken to distinguish the "protein content" of milk as defined above from the "true protein content" which excludes the non-protein nitrogen (NPN) fraction of milk.

4 Principle

Addition of amido black solution, buffered at pH 2,4, to a test portion, resulting in the formation of an insoluble dye-protein complex. Removal of the insoluble complex by centrifuging (or filtration), and determination of the protein content from the absorbance of the resulting solution containing an excess of dye.

5 Reagents

All reagents shall be of recognized analytical grade unless otherwise stated. The water used shall be distilled water or water of at least equivalent purity.

NOTE — Calcium ions interfere with the determination.

5.1 Amido black 10B dyestuff (acid black 1, CI 20470)[1] for milk testing, having a moisture content of less than 5 % (*m*/*m*), or a further purified product.

NOTE — The dyestuff is hygroscopic and should be protected from moisture uptake.

1) Suitable material is available commercially. Details may be obtained from the Secretariat of ISO/TC 34 (MSZH, Hungary) or from ISO Central Secretariat.

5.2 Standard amido black solution.

Prepare a standard solution according to one of the following procedures.

5.2.1 Primary standard, for preparing standard reference solutions.

Introduce into a 1 000 ml volumetric flask containing about 600 ml of water :

— 0,900 g of a purified amido black 10B dyestuff preparation with a high dye content;

— 2,08 g of disodium hydrogenorthophosphate dihydrate ($Na_2HPO_4 \cdot 2H_2O$);

— 15,8 g of citric acid monohydrate ($C_6H_8O_7 \cdot H_2O$).

Dissolve by mechanical agitation for 2 h. Using a pH meter (6.8) check the pH, which shall be 2,40 ± 0,10 at 20 °C; if necessary, adjust the pH by the addition of sulfuric acid solution or sodium hydroxide solution. Dilute to the mark with water and shake for 15 min.

Allow the solution to stand overnight before use. If the solution is to be used over several days, preserve it by adding 0,1 ml of a 5 % solution of thymol in 94 to 97 % (*V*/*V*) ethanol.

From this primary standard, prepare standard matching solutions as described in 5.3.2 and 5.3.3. Determine the absorbance relative to distilled water at a standard wavelength in a standard cell.

5.2.2 Standard solution, for routine determinations in limited numbers.

Completely dissolve in a beaker :

— 0,90 to 0,95 g (depending on the dyestuff content) of the amido black 10B dyestuff (5.1),

— 2,08 g of disodium hydrogenorthophosphate dihydrate ($Na_2HPO_4 \cdot 2H_2O$),

— 15,8 g of citric acid monohydrate ($C_6H_8O_7 \cdot H_2O$),

in 100 to 200 ml of water at 70 to 80 °C, or double or triple quantities of these chemicals and water.

Cool and transfer to a 1 000, 2 000 or 3 000 ml volumetric flask, dilute to about 600, 1 200 or 1 800 ml, respectively, and mix. Adjust the pH to 2,40 ± 0,10 at 20 °C as described in 5.2.1. Dilute to the mark and shake for 15 min. Allow to stand overnight.

Prepare, from this primary solution, the standard matching solutions specified in 5.3.2 and 5.3.3 and determine their absorbances relative to distilled water as described in 5.2.1. Dilute this primary solution with the calculated quantity of citrate buffer solution (5.2.4) to obtain the standard solution which yields standard matching solutions with the same absorbance as those obtained from the primary standard (5.2.1).

Preserve as described in 5.2.1 and allow to stand overnight.

NOTE — As the concentration of amido black may vary from batch to batch, it is necessary that, for each new batch, the concentration be adjusted to that of the primary standard as prepared in 5.2.1, in order that the absorbances of the standard matching solutions prepared in 5.3.2 and 5.3.3 will be the same as those obtained during calibration (see 8.5).

5.2.3 Standard solution, for routine determinations in series.

Completely dissolve, by mechanical agitation for 2 h, in a vessel with a mark (marks) for (an) accurate multiple(s) of 1 000 ml, the corresponding multiple quantities of the chemicals specified in 5.2.2 in a quantity of water which reaches somewhat below the appropriate mark. Adjust the pH to 2,40 ± 0,10 as described in 5.2.1. Dilute to the mark and stir for another 15 min. Allow to stand overnight.

Prepare, from this primary solution, the standard matching solutions specified in 5.3.2 and 5.3.3 and determine their absorbances relative to distilled water as described in 5.2.1. Dilute this primary solution with the calculated quantity of citrate buffer solution (5.2.4) to obtain the standard solution which yields standard matching solutions with the same absorbance as those obtained from the primary standard (5.2.1).

Preserve as described in 5.2.1 and allow to stand overnight.

See the note to 5.2.2.

A final check and adjustment of the concentration should be made using a control sample of milk taken from a large bulk (see 8.6) for which the protein content has been determined by the Kjeldahl reference method (IDF Standard 20).

5.2.4 Buffer solution, for dilution.

Introduce into a 1 000 ml volumetric flask containing about 600 ml of water :

— 2,08 g of disodium hydrogenorthosphosphate dihydrate ($Na_2HPO_4 \cdot 2H_2O$);

— 15,8 g of citric acid monohydrate ($C_6H_8O_7 \cdot H_2O$).

Dissolve by agitation and dilute to the mark with water.

Multiple quantities of these chemicals and water may be taken as required.

Any dilution required shall be carried out with this buffer solution.

5.3 Amido black, standard matching solutions.

Prepare dilutions (standard matching solutions) of the standard amido black solution (5.2) as follows.

5.3.1 Mix 10 volumes of the standard amido black solution with 90 volumes of water by pipetting 10 ml into a 100 ml one-mark volumetric flask and diluting to the mark.

NOTE — This solution is used instead of water to establish the zero scale reading of the spectrometer (6.6), so that scale expansion is obtained and the part of the logarithmic scale where divisions are wider may be used.

5.3.2 Mix 50 volumes of the standard amido black solution with 50 volumes of water by pipetting 50 ml into a 100 ml one-mark volumetric flask and diluting to the mark.

5.3.3 Mix 20 volumes of the standard amido black solution with 80 volumes of water by pipetting 20 ml into a 100 ml one-mark volumetric flask and diluting to the mark.

NOTE — Solutions 5.3.2 and 5.3.3 are equivalent to low and high protein contents and will give corresponding instrumental values. They are used for calibration control (see 8.6.2).

5.4 Standard buffer solutions, for standardization of the pH meter (6.8).

6 Apparatus

All apparatus which may come into contact with the reagents or the test solution shall be made of materials which are not attacked under the conditions of the test and which do not absorb the dyestuff in sufficient quantity to affect the results.

Usual laboratory equipment, and in particular

6.1 Test-tubes or **centrifuge tubes**, fitted with rubber stoppers if necessary.

6.2 Rack, for the test-tubes or centrifuge tubes (6.1).

6.3 Pipette or **syringe**, for milk, to deliver 1,0 ± 0,003 ml (see the note to 6.4).

6.4 Pipette or **syringe**, for the standard amido black solution (5.2), to deliver 20 ± 0,02 ml.

NOTES

1 Pipettes or syringes (6.3 and 6.4) of other capacities may be used, provided that the ratio of the volume of the test portion (see 8.2) to that of the amido black solution (see 8.3.1) is 1 : 20, and that the concentration of amido black in the supernatant liquid (see 8.3.3) is always greater than 0,1 g/l.

2 Any multiple pipetting apparatus used should satisfy the same conditions as those given by the single pipette or syringe method described in this International Standard.

6.5 Mechanical shaker or **rotary mixer** or **compressed-air mixing apparatus**, if required.

6.6 Spectrometer, capable of operating within the wavelength range from 550 to 620 nm, provided with cells (preferably of flowthrough pattern) having optical path lengths of 0,2 to 1,0 mm.

NOTE — It is not necessary to operate at the exact wavelength corresponding to maximum sensitivity as it has been found in practice that calibration compensates for deviations (see 8.4).

6.7 Centrifuge or **filtration apparatus.**

6.7.1 Centrifuge, with swinging cups or racks capable of producing, within at least 2 min, an acceleration of (350 ± 50) g at the lower end of the test-tube (see 10.1).

This acceleration is produced by centrifuges having effective radii (horizontal distances between the centre of the centrifuge spindle and the lower end of the test-tube) and operating at the rotational frequencies indicated in the table.

Table

Effective radius mm	Rotational frequency min^{-1}
240	1 140
245	1 130
250	1 120
255	1 110
260	1 100
265	1 090
270	1 080
275	1 070
300	1 020
325	980

NOTE — The relative centrifugal acceleration produced in a centrifuge is given by the formula

$$1{,}12\, r\, n^2 \times 10^{-6}$$

where

r is the effective horizontal radius, in millimetres;

n is the rotational frequency, per minute.

6.7.2 Filtration apparatus.

Filtration through glass fibre, preferably siliconized, with slight pressure, may be used instead of centrifuging. It is necessary to check that the quantity of dye absorbed by the filter is minimal and is constant for any given surface area. For a given pressure, the porosity of the filter should allow correct filtration of the precipitate formed by the dye-protein complex (see also 10.1).

6.8 pH-meter, fitted with a glass electrode and a suitable reference electrode, allowing measurements of pH to be made to the nearest 0,01 pH unit.

6.9 One-mark volumetric flasks, of capacities 100, 1 000, 2 000 and 3 000 ml.

6.10 Balance, accurate to 0,001 g.

7 Sampling

See ISO 707.

8 Procedure

8.1 Preparation of the test sample

If necessary, adjust the temperature of the sample to 20 to 30 °C by placing the sample in a sample bottle in a water-bath.

Mix the milk thoroughly by gently inverting the sample bottle three or four times, without causing frothing or churning of the fat. If difficulty is experienced in dispersing a cream layer, or if

the milk shows evidence of slight churning, warm the milk slowly to 35 to 40 °C in a water-bath, and mix gently; if necessary, a suitable homogenizer may be used to assist dispersal of the fat.

When uniform distribution of the fat has been obtained, quickly adjust the temperature to 20 °C. Allow the milk to stand for at least 1 min after the final temperature adjustment to allow air bubbles to rise (but not for such a span of time that creaming may occur).

8.2 Test portion

Introduce 1 ml of the prepared test sample (8.1), by means of the pipette or the syringe (6.3), into a test-tube or centrifuge tube (6.1). Mix slightly, shortly before pipetting.

8.3 Determination

8.3.1 Add 20 ml of the standard amido black solution (5.2), by means of the pipette or the syringe (6.4), to the test portion, stopper the tube if necessary, and mix the contents for 30 s by shaking by hand with repeated excursions through about 25 cm or by mechanical means (6.5). Alternatively, use compressed air to effect the mixing.

8.3.2 Remove the stopper if present and separate the protein-dye precipitate by filtration or centrifuging. In the latter case, place the tube in the centrifuge (6.7.1) and centrifuge for at least 2 min after an acceleration of (350 ± 50) g has been reached at the bottom of the tube.

8.3.3 Fill a cell with the supernatant liquid, and measure the absorbance, after having previously adjusted the spectrometer (6.6) to zero absorbance against the amido black standard matching solution (5.3.1) at the selected wavelength (see 6.6).

8.4 Preparation of the calibration curve

Take at least 40 samples from different milks (milks from individual cows if possible) having a total protein content ranging from 2,5 to 4,5 % (m/m) or greater (see the note). Determine the protein content of each using the reference method (IDF Standard 20). If necessary, correct the results for preservation (see 10.1).

In addition, using the method described above, measure the absorbance of the supernatant liquid obtained from each sample.

Plot a graph of protein content obtained by the reference method against absorbance. If there is any departure from a linear relationship at the high protein (low absorbance) end of the curve, then ignore the corresponding results in the calculation of the regression equation. (The procedure for the determination of these high protein contents is described in 10.2.) Also ignore strongly deviating values, i.e. when the protein content determined by the reference method differs by more than twice the standard deviation (deduced from the calculation of the regression equation below) from the corresponding value of the line drawn in the graph.

Deduce the regression equation for the protein content determined by the reference method (y) against absorbance (x) and plot the calibration curve for y against x.

NOTE — Heated (pasteurized and sterilized) and reconstituted milks may require different calibration curves.

8.5 Standard matching solutions

Simultaneously with the preparation of the calibration curve, measure the absorbances of the two standard matching solutions prepared in 5.3.2 and 5.3.3 (see the note to 5.2.2).

8.6 Calibration control

8.6.1 Control milk samples

As the calibration may be affected by seasonal variations of the different nitrogen fractions of milk, it should be frequently checked. This should be carried out using at least one representative sample of a large bulk of milk (i.e. from a large number of cows) of average protein content, or, preferably, using two such samples having high and low protein contents, which have been determined by the Kjeldahl reference method (IDF Standard 20).

8.6.2 Use of control milk samples

Sufficient control samples shall be taken by subsampling bulk milk control samples as described in 8.6.1. A preservative (see 10.1) should be added to these subsamples so that they can be used over a period of days provided that the reference value for the protein content does not change through deterioration.

The control sample or samples should be tested in duplicate prior to each day's testing of routine samples. Normally they should be included in each series of the latter at a frequency of the order of 1 in 10, or interposed at regular intervals during continuous testing for control of "drift". Similarly, it is also necessary to check the spectrometer readings using the standard matching solutions for which the scale readings were determined at the time of calibration (see 8.5 and 8.6.1).

9 Expression of results

9.1 Method of calculation

By means of the calibration curve (see 8.4), deduce the protein content of the sample from the measured absorbance and express the result as grams of protein per 100 g of sample. Correct for any difference between the "Kjeldahl protein content" and the protein content read from the calibration curve of the control milk sample(s) (8.6.1 and 8.6.2).

9.2 Repeatability

The difference between the results of two determinations, carried out in rapid succession by the same analyst, should not exceed 0,03 g of protein per 100 g of sample.

10 Special cases

10.1 Preserved samples

The method is applicable, without modification, to samples preserved by the addition of 0,07 to 0,1 % (*m/m*) of mercury(II) chloride (where pollution regulations permit) or 0,02 to 0,3 % (*m/m*) of sodium azide. For samples preserved with 0,1 % (*m/m*) of potassium dichromate, the method is applicable only if the time between adding the amido black solution to the milk and taking the spectrometric reading is very short, as is the case when using the filtration method (see 6.7); the centrifuging procedure is not applicable. The method is not applicable to samples preserved with formaldehyde.

In the case of preservation with tablets, the presence of about 0,5 % (*m/m*) of sodium chloride is usual. This will affect the absorbance and also the results of the Kjeldahl determination, and correction of the results will be necessary. Deduction of the protein content from a special calibration curve, obtained with such preserved samples and based on Kjeldahl results which have been corrected for the preservation (dilution effect of tablet, nitrogen from azide and tabletting agent), is preferred.

10.2 Departure from linearity in calibration curve

For milks which have high protein contents which do not result in a linear calibration curve, proceed as follows.

Dilute a certain quantity of the sample of the milk with high protein content with the same volume of a (standard, control) milk sample of known protein content, mix well and determine the protein content of the mixture in accordance with 8.3 and 9.1.

Calculate the protein content of the sample of high protein content (w_1) from the protein content of the mixture (w_2) and that of the standard milk (w_3) by means of the equation

$$w_1 = 2w_2 - w_3$$

11 Test report

The test report shall show the method used and the result obtained.

It shall also include

a) the factor used in obtaining the protein content from the nitrogen content determined using the reference method (IDF Standard 20);

b) any observation which may indicate that the result is of doubtful accuracy;

c) all details required for the complete identification of the sample.

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