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Milk — Determination of urea content — Enzymatic method using difference in pH (Reference method)

Lait — Détermination de la teneur en urée — Méthode enzymatique utilisant les fluctuations du pH (Méthode de référence)

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Foreword

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The main task of technical committees is to prepare International Standards, Draft International Standards adopted by the technical committee 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,

Attention is drawn to the possibility that some of the elements of this document may be the subject of patent rights, ISO shall not be held responsible for identifying any or all such patent rights,

International Standard ISO 14637|IDF 195 was prepared by Technical Committee ISO/TC 34, *Food products*, Subcommittee SC 5, *Milk and milk products*, and the International Dairy Federation (IDF), in collaboration with AOAC International. It is being published jointly by ISO and IDF and separately by AOAC International.

Foreword

IDF (the International Dairy Federation) is a worldwide federation of the dairy sector with a National Committee in every member country, Every National Committee has the right to be represented on the IDF Standing Committees carrying out the technical work, IDF collaborates with ISO and AOAC International in the development of standard methods of analysis and sampling for milk and milk products,

Draft International Standards adopted by the Action Teams and Standing Committees are circulated to the National Committees for voting, Publication as an International Standard requires approval by at least 50% of IDF National Committees casting a vote,

International Standard ISO 14637|IDF 195 was prepared by Technical Committee ISO/TC 34, *Food products*, Subcommittee SC 5, *Milk and milk products*, and the International Dairy Federation (IDF), in collaboration with AOAC International. It is being published jointly by ISO and IDF and separately by AOAC International.

All work was carried out by the Joint ISO/IDF/AOAC Action Team, *Nitrogen compounds*, of the Standing Committee on *Main components of milk*, under the aegis of its project leader, Mr Ph. Trossat (FR).

Milk — Determination of urea content — Enzymatic method using difference in pH (Reference method)

1 Scope

This International Standard specifies an enzymatic method for the determination of the urea content of milk by measurement of the difference in pH.

2 Terms and definitions

For the purpose of this document, the following terms and definitions apply.

2.1
urea content
mass fraction of substances determined by the procedure specified in this International Standard

NOTE The urea content is expressed in milligrams per litre.

3 Principle

Urease is added to the test sample to split urea into ammonia and carbon dioxide. At pH 6,7, ammonia immediately hydrolyses thereby releasing hydroxyl ions, and carbon dioxide liberates protons that partly neutralize these hydroxyl ions. The balance between the ammonia and carbon dioxide hydrolysis and the resulting neutralization induces a change in pH. The pH change varies as a function of the urea content of the sample and is measured by using a differential pH analyser.

4 Reagents

Use only reagents of recognized analytical grade, unless otherwise specified, and distilled or demineralized water or water of equivalent purity.

4.1 Reagents for urea determination.

4.1.1 Buffer solution, pH 6,7.

Dissolve 1,777 g of potassium monohydrogenphosphate (K_2HPO_4), 1,388 g of potassium dihydrogen-phosphate (KH_2PO_4), 7,600 g of potassium chloride (KCl), 1,00 g of sodium azide (NaN_3), 0,010 g of acetazolamide (5-acetamido-1,3,4-thiadiazole-2-sulfonamide), 1,040 g of magnesium chloride hexahydrate ($MgCl_2 \cdot 6H_2O$), 2 g of Triton X100, 1 g of Brij 35 and 20 ml of LM1[1] in a 1 000 ml volumetric flask (5.5). Dilute to the mark with water and mix.

1) This detergent is available from Valetudo S.r.l., BG, Italy, and is an example of a suitable product available commercially. This information is given for the convenience of users of this International Standard and does not constitute an endorsement by ISO or IDF of this product.

The buffer solution may be kept for 6 months if stored at 4 °C.

4.1.2 Urease enzyme solution.

Dissolve 360 mg of lyophilized urease (EC 3.5.1.5) in 1 ml of a 50 % (volume fraction) aqueous solution of glycerol. The activity of the obtained urease enzyme solution shall be 2 100 units/ml ± 300 units/ml[2)].

The urease enzyme solution may be kept for 6 months if stored at 4 °C.

4.1.3 Urea standard solution.

Dissolve 1,000 g of dry urea (N_2H_4CO) (dried under vacuum in an oven at 90 °C ± 1 °C for 1 day), 7,45 g of potassium chloride (KCl) and 1,0 g of sodium azide (NaN_3) in a 1 000 ml volumetric flask (5.5). Dilute to the mark with water and mix.

The urea standard solution may be kept for 6 months if stored at 4 °C.

4.1.4 Zero milk.

Add 20 µl of urease solution (4.1.2) to 1 ml of raw milk. Mix and incubate the thus-prepared raw milk for 10 min in the water bath (5.3) set at 40 °C.

4.2 Reagents for cleaning and maintenance of electrodes.

4.2.1 Cleaning solution.

Dissolve 1,742 g of potassium monohydrogenphosphate (K_2HPO_4), 1,361 g of potassium dihydrogen-phosphate (KH_2PO_4), 7,455 g of potassium chloride (KCl), 1,00 g of sodium azide (NaN_3), 2 g of Triton X100, 2 g of Brij 35 and 3 g of LM1[1)] in a 1 000 ml volumetric flask (5.5). Dilute to the mark with water and mix.

The cleaning solution may be kept for 1 year if stored at room temperature.

4.2.2 Regenerating solution.

Use hydrochloric acid of concentration, c(HCl) = 0,1 mol/l.

The regenerating solution may be kept for 1 year if stored at room temperature.

4.2.3 Strong regenerating solution.

Dissolve 30 g of nitric acid (HNO_3) with a mass fraction of approximately 69 %, 30 g of hydrochloric acid (HCl) with a mass fraction of approximately 37 %, 30 g of sodium fluoride (NaF) and 1 g of Triton X100 in a 1 000 ml volumetric flask (5.5). Dilute to the mark with water and mix.

The strong regenerating solution may be kept for 1 year if stored at room temperature.

5 Apparatus

Usual laboratory equipment and, in particular, the following.

5.1 Analytical balance, capable of weighing to the nearest 1 mg.

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2) This unit (often called the International Unit or Standard Unit) is defined as the amount of enzyme which will catalyse the transformation of one micromole of substrate per minute under standard conditions.

5.2 Micropipettes (positive displacement), of capacities 15 µl and 20 µl.

5.3 Water bath, capable of being maintained at 38 °C ± 1 °C and at 40 °C ± 1 °C.

5.4 Differential pH apparatus, generally operating according to the scheme shown in Annex A.

The arrangement and components used may be different.

The differential pH apparatus consists of peristaltic pumps to circulate liquids, a mixing chamber, two glass capillary flow-through electrodes (EL1 and EL2) and an electronic system for measurement.

5.5 One-mark volumetric flasks, of capacity 1 000 ml.

6 Sampling

It is important that the laboratory receive a sample which is truly representative and has not been damaged or changed during transport or storage.

Sampling is not part of the method specified in this International Standard. A recommended sampling method is given in ISO 707.

7 Preparation of test sample

Heat the test sample in the water bath (5.3), set at 38 °C, to that temperature while mixing. Cool to 20 °C just before the preparation of the test portion.

8 Procedure

8.1 General

Since various types of differential pH apparatus (5.4) available differ in design and handling, the operator shall carefully follow the instrument manufacturer's instructions for setting up, calibration and operation of the instrument. Switch the instrument on and allow its operating conditions to stabilize.

If the time between two consecutive measurements is 5 min or more, renew the buffer solution (4.1.1) in the mixing chamber of the apparatus.

8.2 Blank determination

Fill the flow-through electrodes, EL1 and EL2, of the pH apparatus (5.4) with buffer solution (4.1.1). Measure the offset differential pH (D_1) between the electrodes. The difference between the two electrodes shall be between the limits ± 150 mpH (millipH) units.

Using a micropipette (5.2), add 15 µl of urease enzyme solution (4.1.2) to the mixing chamber of the apparatus and mix. Only fill the flow-through electrode EL2 with the buffer/enzyme mixture, Measure again the offset differential pH (D_2) between the two electrodes.

Calculate the difference in pH for the blank, ΔH_0, by using the following equation

$$\Delta H_0 = D_2 - D_1$$

where

ΔH_0 is the difference in pH units between the two offset differential pH measurements, D_1 and D_2, for the blank determination;

D_1 is the numerical value of the differential pH between the electrodes when both are filled with the buffer solution;

D_2 is the numerical value of the differential pH between the electrodes when one is filled with the buffer solution and the other with the buffer/enzyme mixture.

The difference, ΔH_0, between the two electrodes shall be $\pm$ 3 mpH units, while the difference between two consecutive measurements shall be $\leqslant$ 0,5 mpH units.

If these results are not obtained, check the buffer solution and repeat the above procedure. In the case where the results still do not fulfil the requirement(s), than clean the electrodes (see 8.8) and restart the above-mentioned blank determination procedure.

8.3 Calibration

8.3.1 Procedure

Using a micropipette (5.2), add 20 µl of urea standard solution (4.1.3) to the mixing chamber of the pH apparatus (5.4) to a total volume of 1 000 µl (a ratio of volume of urea standard to total volume equal to 1:50 is required). Fill both flow-through electrodes, EL1 and EL2, with the buffer standard mixture. Measure the offset differential pH (D_3).

Using a micropipette (5.2), add 15 µl of urease enzyme solution (4.1.2) to the mixing chamber of the apparatus. Fill electrode EL2 with the buffer standard mixture only. After completion of the enzymatic reaction, measure the offset differential pH (D_4).

8.3.2 Calculation of the pH difference

Calculate the difference in pH for the calibration solution, ΔH_c, by using the following equation

$$\Delta H_c = (D_4 - D_3) - \Delta H_0$$

where

ΔH_c is the difference in pH units between the two offset differential pH measurements D_3 and D_4 (8.3.1) for the calibration determination minus the difference obtained with the blank determination (8.2);

D_3 is the numerical value of the differential pH between the electrodes when both are filled with the buffer standard mixture (8.3.1);

D_4 is the numerical value of the differential pH between the electrodes when one is filled with the buffer solution and the other with the buffer/enzyme mixture (8.3.1).

8.3.3 Calculation of the slope

Calculate the slope, s, of the calibration curve by using the following equation

$$s = \frac{\rho_U}{\Delta H_c}$$

where ρ_U is the concentration of the urea standard solution, expressed in milligrams per litre (4.1.3).

8.4 Checking the calibration

Check the calibration by repeating the blank determination (8.2) and, thereafter, by analysing 20 µl of urea standard solution (4.1.3) by following the procedure in 8.5. The obtained results shall be between the limits ± 15 mg/l for the blank determination and between 970 mg/l and 1 030 mg/l for the urea standard solution. If these results are not obtained, repeat the calibration procedure.

8.5 Determination

Operate the instrument and introduce the test portion according to the manufacturer's instructions.

Using the micropipette (5.2), add 20 µl of test portion to the mixing chamber of the pH apparatus (5.4) to obtain a total volume of 1 000 µl (a ratio of test portion volume to total volume equal to 1:50 is required).

NOTE During analysis, the following process takes place in the instrument. The 20 µl test portion is mixed with the buffer solution (4.1.1). Both flow-through electrodes EL1 and EL2 are filled with the buffer test portion mixture and the differential pH (D_5) between the electrodes is measured. Then a suitable quantity of urease enzyme solution (4.2) is added to the remaining mixture in the mixing chamber and some of the resulting mixture is aspirated into the electrode EL2. The new differential pH (D_6) is monitored after completion of the enzymatic reaction.

8.6 Checking the stability

After analysing 30 test samples and also at the end of the analytical series, analyse two blank solutions to check the zero point and 20 µl of the urea standard solution (4.1.3) by the determination procedure (8.5) to check the calibration.

The second zero value shall be between the limits ± 15 mg/l, and the standard value between the limits 970 mg/l and 1 030 mg/l. If the obtained values are out of this range, repeat the offset blank determination (8.2) and the calibration procedure (8.3).

8.7 Checking the contamination of the electrodes

Check the electrode contamination at least every month by analysing a zero milk sample (4.1.4) using the determination procedure (8.5). The result shall be $\leqslant$ 30 mg/l. If the value obtained is out of this range, operate a strong regeneration (see 9.2) and repeat the calibration procedure (8.3).

8.8 Cleaning procedure

Wash the electrodes and the mixing chamber of the pH apparatus (5.4) with the cleaning solution (4.2.1), replacing the buffer solution (see Figure A.1). If the equipment is in full operation, leave the electrodes in contact with the cleaning solution until the next use, renewing the cleaning solution every 120 min. When not in full operation, treat the electrodes according to the manufacturer's instructions.

9 Maintenance of the electrodes

9.1 Regeneration

At least every week, wash the electrodes and the mixing chamber of the pH apparatus (5.4) with the regenerating solution (4.2.2), thereby replacing the buffer solution (see Figure A.1), followed by the cleaning procedure (8.8).

9.2 Strong regeneration

At least every two months, or earlier if necessary (see 8.4), wash the electrodes and the mixing chamber of the pH apparatus (5.4) with the strong regenerating solution (4.2.3), thereby replacing the buffer solution (see Figure A.1).

NOTE The efficiency of the treatment can be improved by bubbling air through the strong regenerating solution.

After the regeneration, rinse the apparatus with water (see Figure A.1) followed by the cleaning procedure (8.8).

10 Calculation and expression of results

10.1 Calculation

Calculate the urea content of the test sample, w, in milligrams per litre, by using the following equation

$$w = s\left[\left(D_6 - D_5\right) - \Delta H_0\right]$$

where

s is the numerical value of the slope of the calibration curve (8.3.3);

ΔH_0 is the difference in pH units between the two offset differential pH measurements for the blank determination (8.2);

D_5 is the numerical value of the differential pH between the two electrodes when both are filled with the test portion/buffer mixture (8.5);

D_6 is the numerical value of the differential pH between the two electrodes when one is filled with the test portion/buffer mixture and the other with the test portion/buffer/enzyme mixture (8.5).

10.2 Expression of results

Express the test results to one decimal place.

NOTE A conversion of the results to millimoles per litre is possible by application of the coefficient 0,01 665 to the results expressed as milligrams per litre, or a calculation of the slope with the concentration of the urea standard solution expressed in millimoles per litre (mg/l × 0,01 665).

11 Precision

11.1 Interlaboratory tests

Details of two successive interlaboratory tests on the precision of the method are summarized in Annex B.

The values for repeatability and reproducibility limits are expressed for the 95 % probability level and may not be applicable to concentration ranges and matrices other than those given.

11.2 Repeatability

The absolute difference between two individual single test results, obtained with 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, will in not more than 5 % of cases be greater than 15 mg/l.

11.3 Reproducibility

The absolute difference between two single test results, obtained with the same method on identical test material in different laboratories with different operators using different equipment, will in not more than 5 % of cases be greater than 50 mg/l.

12 Test report

The test report shall specify:

a) all information necessary for the complete identification of the sample;

b) the sampling method used, if known;

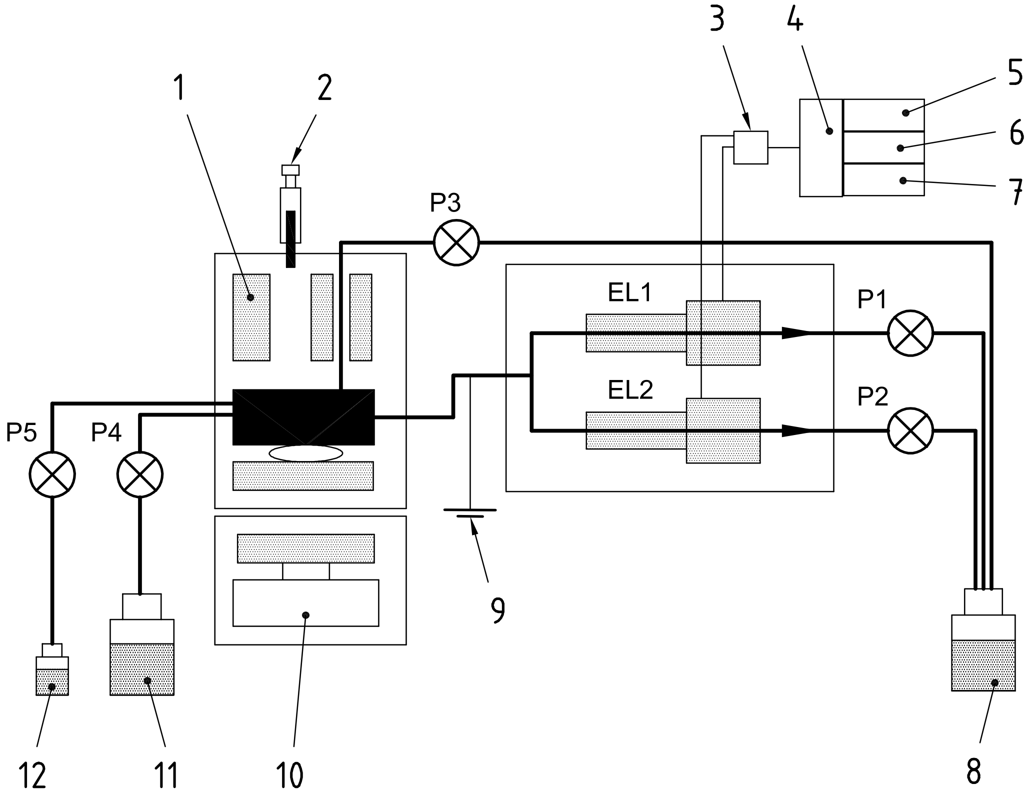
c) the test method used, with reference to this International Standard;

d) all operational 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),

e) the test result(s) obtained and, if the repeatability has been checked, the final quoted result obtained.

Annex A
(informative)

Differential pH apparatus



Key

1 mixing chamber

2 micropipette for injection of sample

3 differential amplifier

4 electronics

5 printer

6 display

7 keyboard

8 waste

9 ground

10 magnetic stirrer

11 buffer solution

12 enzyme solution

EL1 and EL2 are glass capillary electrodes

P1 to P5 are peristaltic pumps

Figure A.1 — Example of a differential pH apparatus

Annex B
(informative)

Interlaboratory tests

An interlaboratory collaborative test involving 18 laboratories was carried out on 10 test samples with two replicates. The results are given in Reference [4].

The test results were subject to statistical analysis in accordance with ISO 5725-1 and ISO 5725-2 to give the precision data shown in Tables B.1 and B.2.

Table B.1 — First interlaboratory test

Values are in milligrams per litre

Sample	Mean	s_L	s_r	s_R	CV(r) %	CV(R) %	r	R
1	217,0	11,9	4,2	12,7	1,95	5,84	11,9	35,7
2	253,2	13,8	4,8	14,6	1,91	5,78	13,8	41,3
3	291,1	15,8	2,9	16,0	0,99	5,48	8,3	45,2
4	329,4	15,3	4,3	16,0	1,31	4,85	12,2	45,1
5	368,7	17,5	3,7	17,9	1,01	4,86	10,5	50,8
6	410,7	11,4	7,2	13,6	1,76	3,31	20,5	38,4
7	446,9	17,7	3,7	18,1	0,83	4,06	10,5	51,3
8	486,8	16,1	6,5	17,3	1,33	3,55	18,2	48,9
9	526,9	20,0	3,4	20,3	0,65	3,85	9,6	57,5
10	563,0	20,1	9,1	22,0	1,61	3,92	25,6	62,4
Overall mean					1,40	4,64		

s_L: between-laboratories standard deviation
s_r : repeatability standard deviation
s_R: reproducibility standard deviation
r: repeatability limit
R: reproducibility limit
CV(r): repeatability coefficient of variation
CV(R) reproducibility coefficient of variation

A second interlaboratory collaborative test involving 23 laboratories was carried out on 10 test samples with two replicates.

Table B.2 —Second interlaboratory test

Values are in milligrams per litre

Sample	Mean	s_L	s_r	s_R	CV(r) %	CV(R) %	r	R
1	167,9	12,3	4,1	13,0	2,45	7,73	11,6	36,7
2	187,7	10,1	2,5	10,4	1,32	5,54	7,0	29,5
3	235,0	13,7	4,3	14,3	1,84	6,09	12,2	40,5
4	266,4	13,3	3,5	13,7	1,31	5,14	9,8	38,8
5	307,0	13,1	5,0	14,0	1,64	4,56	1,4	39,7
6	344,2	13,3	3,5	13,8	1,02	4,01	10,0	39,0
7	380,8	13,1	5,2	14,1	1,35	3,71	14,6	39,9
8	415,4	13,5	4,0	14,0	0,97	3,37	11,3	39,8
9	450,2	13,9	3,4	14,2	0,75	3,16	9,6	40,4
10	487,2	15,0	3,6	15,5	0,74	3,17	10,3	43,7
Overall mean					1,43	4,86		

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[1] ISO 707, *Milk and milk products — Guidance on sampling*[3)]

[2] ISO 5725-1, *Accuracy (trueness and precision) of measurement methods and results — Part 1: General principles and definitions*

[3] ISO 5725-2, *Accuracy (trueness and precision) of measurement methods and results — Part 2: Basic method for the determination of repeatability and reproducibility of a standard measurement method*

[4] LUZZANA M. and GIARDINO R. Urea determination in milk by a differential pH technique. *Le Lait*, **79**, 1999, pp. 261-267

3) Equivalent to IDF 50 C:1995.

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