INTERNATIONAL STANDARD

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Cheese and processed cheese products — Determination of citric acid content — Enzymatic method

Fromages et fromages fondus — Détermination de la teneur en acide citrique — Méthode enzymatique

Reference number
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Foreword

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International Standard ISO 2963 was prepared by Technical Committee ISO/TC 34, *Agricultural food products*, Subcommittee SC 5, *Milk and milk products*, in collaboration with the International Dairy Federation (IDF) and the AOAC INTERNATIONAL, and will also be published by these organizations.

This second edition cancels and replaces the first edition (ISO 2963:1974), which has been technically revised.

Annex A forms an integral part of this International Standard. Annexes B and C are for information only.

International Organization for Standardization
Case postale 56 • CH-1211 Genève 20 • Switzerland
Internet central@iso.ch
X.400 c=ch; a=400net; p=iso; o=isocs; s=central
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Cheese and processed cheese products — Determination of citric acid content — Enzymatic method

1 Scope

This International Standard specifies an enzymatic method for the determination of the citric acid content of cheese and processed cheese products.

CAUTION – Reliable results will only be obtained if the Good Laboratory Practice (GLP) rules for enzymatic analyses are applied strictly. These GLP rules are given in annex A.

2 Definition

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

2.1 citric acid content: Mass fraction of substances, determined by the procedure specified in this International Standard. It is expressed as a percentage by mass.

3 Principle

Treatment of an extract of the sample with the following enzymes and biochemical substances:

— citrate lyase (CL) to convert citric acid to oxaloacetate and acetate;

— malate dehydrogenase (MDH) and lactate dehydrogenase (LDH) in the presence of reduced nicotinamide-adenine dinucleotide (NADH) to catalyse the reduction of oxaloacetate and its decarboxylation product, pyruvate, to L-malate and L-lactate, respectively, with subsequent conversion of NADH to this oxidized form (NAD^+).

Determination of the decrease in concentration of NADH by measurement of the absorbance of the test solution at 340 nm. The citric acid content is proportional to the decrease in NADH concentration.

4 Reagents

Use only reagents of recognized analytical grade, unless otherwise specified, and (glass-) distilled or demineralized water or water of equivalent purity. Use double (glass-) distilled water for the preparation of the enzyme solutions.

4.1 Trichloroacetic acid solution

Dissolve 200 g of trichloroacetic acid (CCl_3COOH) in water, and dilute with water to 1 000 ml. Mix the solution.

4.2 Sodium hydroxide solution A, c(NaOH) = 5,0 mol/l.

Dissolve 200,0 g of sodium hydroxide in water in a 1 000 ml volumetric flask (5.5), and dilute to 1 000 ml with water. Mix the solution.

4.3 Sodium hydroxide solution B, c(NaOH) = 1,0 mol/l.

Dissolve 40,0 g of sodium hydroxide in water in a 1 000 ml volumetric flask (5.5), and dilute to 1 000 ml with water. Mix the solution.

4.4 Sodium hydroxide solution C, $c(NaOH)$ = 0,1 mol/l.

Dissolve 4,0 g of sodium hydroxide in water in a 1 000 ml volumetric flask (5.5), and dilute to 1 000 ml with water. Mix the solution.

4.5 Zinc chloride solution ($ZnCl_2$)

Dissolve 0,80 g of zinc chloride in water in a 1 000 ml volumetric flask (5.5), and dilute to 1 000 ml with water. Mix the solution.

4.6 Buffer solution, pH = 7,8.

Dissolve 71,3 g of glycylglycine ($H_2NCH_2CONHCH_2CO_2H$) in about 700 ml of water in a 1 000 ml volumetric flask (5.5). Adjust the pH to 7,8 with sodium hydroxide solution A (4.2). Add 100 ml of the zinc chloride solution (4.5) and dilute to 1 000 ml with water. Mix the solution.

If stored in a refrigerator at between 0 °C and + 8 °C, the solution can be kept for 4 weeks.

4.7 Sodium hydrogen carbonate ($NaHCO_3$)

Dissolve 4,0 g of sodium hydrogen carbonate in water in a 1 000 ml volumetric flask (5.5), and dilute to 1 000 ml with water. Mix the solution.

4.8 Reduced nicotinamide-adenine dinucleotide solution

Dissolve 50 mg of reduced nicotinamide-adenine dinucleotide disodium salt ($C_{21}H_{27}N_7O_{14}P_2Na_2$) and 100 mg of sodium hydrogen carbonate (4.7) in 10 ml of water.

If stored in a refrigerator at between 0 °C and + 8 °C, the solution can be kept for 4 weeks.

4.9 Ammonium sulfate solution, $c\left[(NH_4)_2SO_4\right]$ = 3,2 mol/l.

Dissolve 422,4 g of ammonium sulfate in water in a 1 000 ml volumetric flask (5.5), and dilute to 1 000 ml with water. Mix the solution.

4.10 Malate dehydrogenase/lactate dehydrogenase suspension

Mix sufficient malate dehydrogenase [(MDH) from pig heart; suspension in ammonium sulfate solution (4.9); pH about 6; EC 1.1.1.37][1] and lactate dehydrogenase [(LDH) from rabbit muscle; suspension in ammonium sulfate solution (4.9); pH about 7; EC 1.1.1.27][1] and dilute with the ammonium sulfate solution so as to obtain a suspension containing about 600 units[2] of MDH per millilitre and 1 400 units of LDH per millilitre.

If stored in a refrigerator at 0 °C to + 8 °C, the suspension can be kept for 1 year.

4.11 Citrate lyase solution

Dissolve sufficient citrate lyase [Lyophilisate (CL) from *Aerobacter aerogenes*; EC 4.1.3.6] in ice-cold water, so as to obtain a solution containing 40 units of CL per millilitre.

If stored in a refrigerator, the solution can be kept for 1 week at between 0 °C and + 8 °C. If stored at – 20 °C, the solution can be kept for 4 weeks.

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[1] The EC number refers to the Enzyme Classification Number as given in reference [1].

[2] Unit (often called International or Standard Unit) is defined as the amount of enzyme which will catalyse the transformation of 1 μmol of substrate per minute under standard conditions.

4.12 Citric acid standard solution

Dissolve 1,600 g of citric acid monohydrate ($C_6H_8O_7 \cdot H_2O$) in water in a 1 000 ml volumetric flask (5.5), and dilute with water to 1 000 ml. Mix the solution.

It is important to take account of the production and expiry dates of the reagents given by the manufacturer.

NOTES

1 If an enzyme suspension is applied with other than the specified activity, the volume of the suspension as stated in the pipetting scheme (8.5.1) should be increased or decreased proportionally.

2 The reagents as described in 4.5 to 4.12 inclusive may be obtained commercially as a test combination.

5 Apparatus

Usual laboratory equipment and, in particular, the following.

5.1 Analytical balance, capable of weighing to within 0,1 mg.

5.2 pH-meter, accurate to within ± 0,1 pH unit at 25 °C.

5.3 Glass beakers, of capacity 50 ml.

5.4 Macerator, with suitable beaker (Ultra Turax[3] or equivalent is suitable).

5.5 One-mark volumetric flasks, of capacity 1 000 ml and 100 ml.

5.6 Pipettes, to deliver 25 ml, 10 ml, 5 ml, 2 ml, 1 ml, 0,1 ml and 0,02 ml, respectively.

5.7 Graduated pipettes, to deliver 10 ml, graduated in 0,1 ml divisions.

5.8 Measuring cylinder, of capacity 50 ml.

5.9 Filter funnel, of diameter about 7 cm.

5.10 Filter paper, medium grade, of diameter about 15 cm.

5.11 Spectrometer, capable of measuring at a wavelength of 340 nm, equipped with plastic, glass or quartz **cells** of optical path length 1 cm.

5.12 Plastic paddles, capable of mixing the test sample/enzyme mixture in the spectrometer cell.

5.13 Water bath, capable of being maintained at between 20 °C and 25 °C, with rack suitable for holding the spectrometer cell (5.11) during the incubation period (optional; see 8.5.1).

NOTE 3 Incubation of the cells in the water bath is only necessary if the room temperature is below 20 °C.

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[2].

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[3] Ultra-Turax 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 of this product.

7 Preparation of test sample

7.1 Prepare an homogeneous test sample taking care to avoid loss of moisture, using one of the following procedures.

a) **Cheese**
Remove the rind or mouldy surface layer of the cheese in such a way as to provide a sample representative of the cheese as it is usually consumed. Grind or grate the sample by means of an appropriate device. Mix the ground or grated mass quickly and, if possible, grind or grate a second time and again mix thoroughly by intensive stirring and kneading.

b) **Processed cheese**
Remove a sample representative of the product. Mix the sample mass quickly and grind it, if necessary, by means of an appropriate device. Mix thoroughly by intensive stirring and kneading.

c) **Processed cheese containing pieces of other foods** (e.g. ham, fruit, nuts, herbs)
Determine whether the objective of the analysis is to determine the citric acid content of the processed cheese only or of the entire product. Proceed with the entire product as for processed cheese, b). In the former case, separate the pieces of other food and then proceed as for processed cheese, b).

7.2 Transfer the test sample to a container provided with an airtight lid, for storage prior to analysis. Close the container immediately. Analysis should be carried out as soon as possible after preparation of the test sample.

8 Procedure

NOTE 4 If it is required to check whether the repeatability is met, carry out two single determinations in accordance with 8.2, 8.4 and 8.5 under repeatability conditions.

8.1 Check test

8.1.1 Carry out the following test to check the recovery of citric acid whenever a new batch of reagents (4.5 to 4.12) is brought into use, or when such reagents have been kept in a refrigerator without being used for more than 2 weeks, or when restarting analytical work after a period of analytical inactivity, or whenever conditions may justify such a test.

8.1.2 Pipette into each of two 100 ml volumetric flasks (5.5), 5,0 ml and 10,0 ml of the citric acid standard solution (4.12) respectively. Add to each flask 10 ml of the trichloroacetic acid solution (4.1). Dilute the contents of each to 100 ml and swirl to mix. Determine the citric acid content of both solutions as described in 8.4.3 to 8.5.3 inclusive.

The test shall be repeated until satisfactory results are obtained.

8.2 Test portion

Weigh 1 g or more of the prepared test sample (clause 7), to the nearest 0,1 mg, into the macerator beaker (5.4).

8.3 Blank test

Carry out a blank test in duplicate, proceeding as specified in 8.4 and 8.5, using all reagents but omitting the test portion.

8.4 Preparation of suspension and deproteination

8.4.1 Suspend the test portion in about 50 ml of warm water (40 °C to 50 °C) using the macerator (5.4). Transfer the contents of the beaker quantitatively into a 100 ml volumetric flask (5.5). Cool the contents of the flask to about 20 °C.

8.4.2 Add to the suspension 10 ml of the trichloroacetic acid (4.1). Dilute to the mark with water, mix thoroughly and let the mixture stand for 30 min. Do not remix the contents of the volumetric flask prior to filtration.

8.4.3 Filter the supernatant liquid through a filter paper (5.10), discarding the first few millilitres of the filtrate.

8.4.4 Pipette 25 ml of the filtrate into a beaker (5.3) and adjust the pH to approximately 4 by addition of the sodium hydroxide solution B (4.3) and subsequently to approximately 8 by addition of the sodium hydroxide solution C (4.4), using the pH-meter (5.2).

Transfer the contents of the beaker quantitatively to a 100 ml volumetric flask (5.5). Dilute to the mark with water and mix.

8.4.5 Filter through a filter paper (5.10), discarding the first few millilitres of the filtrate.

8.5 Determination

8.5.1 Carry out the determination according to the following scheme, taking care to bring the buffer solution (4.6) and the water to be used to room temperature just before use.

a) Pipette into the spectrometer cells:

	For determination of test portion or for check test	For blank test
Buffer sollution (4.6)	1,00 ml	1,00 ml
NADH solution (4.8)	0,10 ml	0,10 ml
MDH/LDH suspension (4.10)	0,02 ml	0,02 ml
Test or check-test filtrate	2,00 ml	–
Blank-test filtrate	–	2,00 ml

b) Mix the contents of the cells, using the plastic paddles (5.12) and incubate the solutions in the water bath (5.13) for 5 min at a temperature between 20 °C and 25 °C. Measure the absorbance A_0 of the solution in each cell, against air, using the spectrometer (5.11) at a wavelength of 340 nm.

c) Add 0,02 ml citrate lyase (4.7) for the determination of the test portion or for the check test or the blank test.

d) Mix the contents of the cells and incubate the solutions in the water bath (5.13) for 10 min at a temperature between 20 °C and 25 °C.

e) Measure the absorbance A_{10} of the solution in each cell, against air.

8.5.2 Calculate the absorbance A of each cell content to be used for the calculation of citric acid content (9.1) by means of equation (1)

$$A = A_0 - A_{10} \quad ...(1)$$

where

A_0 is the absorbance measured before addition of the citrate lyase;

A_{10} is the absorbance measured after the addition of citrate lyase and incubation for 10 min.

8.5.3 If the decrease in absorbance exceeds 0,800, repeat the procedure specified in 8.5.1 and 8.5.2, using an appropriate aqueous dilution of the filtrate from both the test portion (8.4) and the blank test (8.3).

9 Calculation and expression of results

9.1 Check test

9.1.1 Calculate the content of citric acid monohydrate, w_1, of the standard solution (4.12) according to equation (2), using the following values:

M = 210,1 g/mol (molar mass of citric acid monohydrate)

V_5 = 1 000 ml (4.12)

V_6 = 5 ml and 10 ml respectively (see 8.1.2)

V_7 = 100 ml (see 8.1.2)

9.1.2 Taking into account the purity of the citric acid monohydrate, the recovery obtained for both dilutions (8.1.2) shall be within the range (100 ± 5) %.

If the recoveries are not within this range, the reagents, operating technique, accuracy of the pipettes and the condition of the spectrometer shall be checked and the required action shall be taken to obtain the appropriate results.

9.2 Test portion

Calculate the content of citric acid, w, expressed as a percentage by mass of anhydrous citric acid, using equation (2):

$$w = \frac{(A_s - A_r)M}{k\,l\,m} \times \frac{V_1\,V_3\,V_5}{V_2 V_4} \times \frac{V_7}{V_6} \times 100\ \%$$

$$= \frac{1{,}915(A_s - A_r)}{m} \times \frac{V_7}{V_6}\ \% \qquad \ldots(2)$$

where

A_s is the numerical value of the absorbance measured for the test portion or the check test;

A_r is the numerical value of the average absorbance measured for the blank test;

M is the numerical value of the molar mass of citric acid (for anhydrous citric acid, M = 192,1 g/mol);

k is the numerical value of the molar absorption coefficient of NADH at 340 nm (i.e. $6{,}3 \times 10^6$ cm^2/mol);

l is the numerical value of the optical path length of the spectrometer cells (l = 1 cm);

m is the numerical value of the mass, in grams, of the test portion (8.2);

V_1 is the numerical value of the total volume of liquid in the spectrometer cell (V_1 = 3,14 ml);

V_2 is the numerical value of the volume of filtrate (8.4.5) in the spectrometer cell (V_2 = 2,00 ml);

V_3 is the numerical value of the volume to which the deproteinated, filtered solution (8.4.3) was diluted after adjusting the pH to 8 (8.4.4) (V_3 = 100 ml);

V_4 is the numerical value of the volume of the deproteinated, filtered solution (8.4.3) taken for adjusting the pH to 8 (8.4.4) (V_4 = 25 ml);

V_5 is the numerical value of the volume of the solution in 8.4.2 (V_5 = 100 ml);

V_6 is the numerical value of the volume, in millilitres, of the filtrate (8.4.5) taken for dilution (8.5.3), if appropriate;

V_7 is the numerical value of the volume, in millilitres, to which the test solution (see 8.4.5) was diluted (see 8.5.3), if appropriate.

9.3 Expression of results

Express the results to two decimal places. Results of 1 % or less should be expressed to three decimal places.

10 Precision

The values for repeatability and reproducibility have been derived from the results of an interlaboratory test carried out in accordance with ISO 5725[3][4]. These results are reported in annex B.

10.1 Repeatability

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

10.2 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, should not be greater than 8 % of the arithmetic mean of the results.

11 Test report

The test report shall specify:

– the method in accordance with which sampling was carried out, if known;

– the method used;

– the result obtained; and

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

It shall also mention all operating conditions 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.