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Determination of substances characteristic of green and black tea —

Part 1:
Content of total polyphenols in tea — Colorimetric method using Folin-Ciocalteu reagent

Détermination des substances caractéristiques du thé vert et du thé noir —

Partie 1: Dosage des polyphénols totaux dans le thé — Méthode colorimétrique utilisant le réactif de Folin-Ciocalteu

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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 preparing International Standards is normally carried out through ISO technical committees. Each member body interested in a subject for which a technical committee has been established 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. ISO collaborates closely with the International Electrotechnical Commission (IEC) on all matters of electrotechnical standardization.

International Standards are drafted in accordance with the rules given in the ISO/IEC Directives, Part 2.

The main task of technical committees is to prepare International Standards. 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.

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.

ISO 14502-1 was prepared by Technical Committee ISO/TC 34, *Food products*, Subcommittee SC 8, *Tea*.

ISO 14502 consists of the following parts, under the general title *Determination of substances characteristic of green and black tea*:

— *Part 1: Content of total polyphenols in tea — Colorimetric method using Folin-Ciocalteu reagent*

— *Part 2: Content of catechins in green tea — Method using high-performance liquid chromatography*

Determination of substances characteristic of green and black tea —

Part 1:
Content of total polyphenols in tea — Colorimetric method using Folin-Ciocalteu reagent

1 Scope

This part of ISO 14502 specifies a method for the determination of the total polyphenol content of leaf tea and instant tea by a colorimetric assay using Folin-Ciocalteu phenol reagent[4]. It is applicable to both green and black tea products.

2 Normative references

The following referenced documents are indispensable for the application of this document. For dated references, only the edition cited applies. For undated references, the latest edition of the referenced document (including any amendments) applies.

ISO 1572, *Tea — Preparation of ground sample of known dry matter content*

ISO 3696:1987, *Water for analytical laboratory use — Specification and test methods*

ISO 7513, *Instant tea in solid form — Determination of moisture content (loss in mass at 103 °C)*

3 Principle

Polyphenols are extracted with 70 % methanol from a test portion of finely ground leaf tea at 70 °C. Instant teas are dissolved in hot water with 10 % acetonitrile (volume fraction) added to stabilize the extract. The polyphenols in the extract are determined colorimetrically using Folin-Ciocalteu phenol reagent. The reagent contains phospho-tungstic acids as oxidants, which on reduction by readily oxidized phenolic hydroxy groups yield a blue colour with a broad maximum absorption at 765 nm. This is due to the formation of so-called tungsten and molybdenum blues. The Folin-Ciocalteu phenol reagent reacts with a wide range of polyphenol compounds and, although the response can vary with the individual components, selection of gallic acid as a calibration standard enables useful total polyphenol data to be obtained.

4 Reagents

Use only reagents of recognized analytical grade, unless otherwise specified.

4.1 Water, conforming to grade 1 of ISO 3696:1987.

4.2 Acetonitrile.

4.3 Methanol.

4.4 Methanol/water extraction mixture, 70 % methanol (volume fraction).

Add 700 ml of the methanol (4.3) to a 1 litre one-mark volumetric flask. Dilute to the mark with water (4.1) and mix.

4.5 Folin-Ciocalteu phenol reagent, commercially available ready prepared.

It is advisable to check the calibration linearity with respect to gallic acid in order to ascertain the suitability of the supplied reagent.

4.6 Dilute Folin-Ciocalteu phenol reagent, 10 % (volume fraction).

Using a pipette, transfer 20 ml of Folin-Ciocalteu phenol reagent (4.5) to a 200 ml one-mark volumetric flask. Dilute to the mark with water and mix.

Prepare fresh reagent solution daily.

To avoid contamination of the concentrated Folin-Ciocalteu reagent, discard any unused dispensed reagent.

4.7 Sodium carbonate solution, 7,5 % (mass concentration).

Weigh (37,50 ± 0,01) g of anhydrous sodium carbonate (Na_2CO_3) into a 500 ml one-mark volumetric flask. Add sufficient warm water to half-fill the flask. Swirl to dissolve the sodium carbonate, cool to room temperature, dilute to the mark with water and mix.

NOTE This solution will remain stable at room temperature for up to 1 month.

4.8 Gallic acid stock standard solution, corresponding to approximately 1 000 µg/ml of anhydrous gallic acid.

Weigh (0,110 ± 0,001) g of gallic acid monohydrate (M = 188,14) into a 100 ml one-mark volumetric flask. Dissolve in water, dilute to the mark and mix.

Prepare a fresh standard solution daily.

NOTE Gallic acid monohydrate is preferred over anhydrous, due to its greater solubility, reduced hygroscopic properties and availability of certified reagent grades, e.g. A.C.S., which is used to denote chemicals that meet specifications outlined by the American Chemical Society. If not known, the moisture content (loss in mass at 103 °C) on a portion of the standard material should be determined. The concentration of the stock standard solution on a gallic acid anhydrous basis can then be calculated.

4.9 Gallic acid standard solutions A to E

Using pipettes, transfer the volumes of gallic acid stock standard solution (4.8) given in Table 1 to 100-ml one-mark volumetric flasks. Dilute to the mark with water and mix. These dilute standard solutions should be prepared on the day of use.

Table 1 — Gallic acid dilute standard solutions

Gallic acid standard solution	Volume of gallic acid stock solution ml	Nominal concentration of dilute standard µg/ml
A	1,0	10
B	2,0	20
C	3,0	30
D	4,0	40
E	5,0	50

5 Apparatus

Usual laboratory apparatus and, in particular, the following.

5.1 Analytical balance, capable of weighing to an accuracy of ± 0,001 g.

5.2 Water bath, capable of being maintained at 70 °C ± 1 °C.

5.3 Dispenser, for methanol/water extraction mixture (4.4), and set at 5,0 ml.

5.4 Centrifuge, capable of 2 000 Relative Centrifugal Force (R.C.F.) (typically 3 500 r/min).

5.5 Spectrophotometer, set at 765 nm and able to accommodate 10 mm path length cells, preferably in an automatic flow cell assembly.

5.6 Pipettes, glass or automatic, to cover the volume range for standard and sample extract dilutions.

5.7 One-mark volumetric flasks, of capacities 100 ml, 200 ml, 500 ml, and 1 litre.

5.8 Vortex mixer, for efficient mixing during extraction.

5.9 Extraction tubes, glass, of 10 ml capacity, stoppered and able to withstand being centrifuged.

5.10 Graduated tubes, glass, of 10 ml capacity with 0,1 ml graduations.

As the assay is sensitive to traces of organic impurities, extraction tubes (5.9) and graduated tubes (5.10) should all be taken through an additional cleaning procedure of washing in approx. 15 % (volume fraction) nitric acid, followed by rinsing thoroughly in water and drying in an air oven at 100 °C. The use of disposable plastic tubes as an alternative to the graduated tubes in the final colorimetic assay is recommended, as additional cleaning has not been found necessary.

6 Sampling

A representative sample should have been sent to the laboratory. It should not have been damaged or changed during transport or storage.

Sampling is not part of the method specified in this part of ISO 14502. A recommended sampling method is given in

— ISO 1839 for leaf tea, and

— ISO 7516 for instant tea.

7 Preparation of test samples

To ensure homogeneity, grind the sample of leaf tea in accordance with ISO 1572 and store samples in well-sealed containers protected from light.

Grinding of instant tea is only required on samples of a coarse granular structure.

8 Procedure

8.1 General

If it is required to check whether the repeatability limit (10.2) is met, carry out two single determinations in accordance with 8.2 to 8.6 under repeatability conditions.

8.2 Determination of dry matter content

Calculate the dry matter content from the moisture content (loss in mass at 103 °C) determined on a portion of the test sample (Clause 7) in accordance with

— ISO 1572 for leaf tea, or

— ISO 7513 for instant tea.

8.3 Test portion

8.3.1 Instant tea

Weigh (0,500 ± 0,001) g of the test sample (Clause 7) into a 50 ml one-mark volumetric flask.

8.3.2 Leaf tea

Weigh (0,200 ± 0,001) g of the test sample (Clause 7) into an extraction tube (5.9).

8.4 Extraction of polyphenols

8.4.1 Instant tea

8.4.1.1 Add to the instant tea in the flask (8.3.1) approximately 25 ml of hot water (maximum temperature 60 °C). Mix to dissolve the sample and allow to cool to room temperature.

8.4.1.2 Add 5,0 ml of acetonitrile (4.2). Dilute to the mark with water and mix.

8.4.2 Leaf tea

8.4.2.1 Place the methanol/water extraction mixture (4.4) contained in the dispenser (5.3) in the water bath (5.2) set at 70 °C, and allow at least 30 min for the extraction mixture to equilibrate.

8.4.2.2 Place the extraction tube containing the sample (8.3.2) in the water bath set at 70 °C. Dispense 5,0 ml of hot methanol/water extraction mixture from 8.4.2.1 into the extraction tube, stopper and mix on the vortex mixer (5.8).

8.4.2.3 Continue heating the extraction tube in the water bath for 10 min, mixing on the vortex mixer after 5 min and 10 min.

It is important to mix the samples thoroughly to ensure complete extraction.

8.4.2.4 Remove the extraction tube from the water bath and allow it to cool to room temperature. Remove the stopper and place the tube in the centrifuge (5.4) at 3 500 r/min for 10 min.

8.4.2.5 Carefully decant the supernatant into a graduated tube (5.10).

8.4.2.6 Repeat extraction steps 8.4.2.2 to 8.4.2.5. Combine the extracts, make up to 10 ml with cold methanol/ water extraction mixture (4.4) and mix the contents.

8.4.2.7 The extract from 8.4.2.6 is stable for at least 24 h if stored at 4 °C. Allow the extract to attain room temperature before proceeding with the assay. Resuspension of the small amount of particulate material that may settle during storage is not necessary.

8.5 Dilution

Using a pipette, transfer 1,0 ml of the sample extract (instant tea extract from 8.4.1.2 or leaf tea extract from 8.4.2.6) into a one-mark 100 ml volumetric flask. Dilute to the mark with water and mix.

8.6 Determination

8.6.1 Using a pipette, transfer 1,0 ml of the gallic acid standard solutions A, B, C, D and E (4.9), in duplicate, into separate plastic or graduated tubes (5.10).

NOTE These correspond to approximately 10 µg, 20 µg, 30 µg, 40 µg and 50 µg of anhydrous gallic acid.

8.6.2 Using a pipette, transfer 1,0 ml of water, in duplicate, into separate tubes (5.10). These are reagent blanks.

8.6.3 Using a pipette, transfer 1,0 ml of diluted sample extract (8.5), in duplicate, into separate tubes.

8.6.4 Using a pipette, add 5,0 ml of dilute Folin-Ciocalteu phenol reagent (4.6) into each tube and mix.

8.6.5 Within 3 min to 8 min after the addition of the dilute Folin-Ciocalteu phenol reagent, pipette 4,0 ml of sodium carbonate solution (4.7) into each tube. Stopper and mix.

8.6.6 Allow to stand at room temperature for 60 min, and then measure the optical densities in 10-mm path length cells against water on the spectrophotometer (5.5) set at 765 nm.

8.6.7 The reagent blank (8.6.2) should have an optical density of < 0,010. Higher values indicate contamination problems from poor quality water, reagents or glassware. It is also important that the sample optical density be within the calibration range. Repeat the colorimetric assay with an increased dilution (8.5) if the sample optical density is higher than the 50 µg gallic acid standard E.

9 Calculation

9.1 Calculate, to the nearest 0,1 µg, the mass of anhydrous gallic acid, m, in the 1,0 ml aliquots of the standard solutions A, B, C, D and E (4.9) taken in 8.6.1, using the formula:

$$m = \frac{m_0 \times V \times w_{\text{DM, std}} \times 10\ 000}{100 \times 100}$$

where

m_0 is the mass of gallic acid monohydrate, in grams, used to prepare the stock standard solution (4.8);

V is the volume of gallic acid stock standard solution, in millilitres, used to prepare the standard solutions A, B, C, D and E (4.9);

$w_{\text{DM, std}}$ is the dry matter content, expressed as a mass fraction, in percent, of the gallic acid.

9.2 Construct a best-fit linear calibration graph from the mass of anhydrous gallic acid in standards A, B, C, D and E (4.9), as calculated in 9.1, against the gallic acid standard optical densities after subtracting the reagent blank optical density.

Obtain the calibration line slope and intercept value. A typical gallic acid calibration graph is given in Annex A.

A linear calibration should be obtained. Calculate the calibration line slope value to the nearest 0,000 1 for use in subsequent calculations. The linear calibration should also have an intercept close to the origin. Intercept values on the optical density y-axis $> \pm 0{,}04$ should be investigated. For example, check the gallic acid moisture content, standard preparation or pipette calibration.

The total polyphenol content, w_T, expressed as a percentage by mass on a sample dry matter basis, is given by the formula:

$$w_T = \frac{\left(D_{sample} - D_{intercept}\right) \times V_{sample} \times d \times 100}{S_{std} \times m_{sample} \times 10\ 000 \times w_{DM,\ sample}}$$

where

D_{sample} is the optical density obtained for the sample test solution;

$D_{intercept}$ is the optical density at the point the best-fit linear calibration line intercepts the y-axis;

S_{std} is the slope obtained from the best-fit linear calibration;

m_{sample} is the mass, in grams, of the sample test portion;

V_{sample} is the sample extraction volume, in millilitres (50 ml for instant tea and 10 ml for leaf tea);

d is the dilution factor used prior to the colorimetric determination (typically 1,0 ml to 100 ml, thus a dilution factor of 100);

$w_{DM,\ sample}$ is the dry matter content, expressed as a mass fraction in percent, of the test sample, determined in accordance with 8.2.

10 Precision

10.1 Interlaboratory test

Details of the interlaboratory test to determine the precision of the method are summarized in Annex B. The values derived from this interlaboratory test may not be applicable to concentration ranges and matrices other than those given.

10.2 Repeatability

The absolute difference between two independent single test results, obtained using 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 the repeatability limit values, r, given in Table B.1.

10.3 Reproducibility

The absolute difference between two single test results, obtained using 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 the reproducibility limit values, R, given in Table B.1.

11 Test report

The test report shall specify:

— all information necessary for the complete identification of the sample;

— the sampling method used, if known;

— the test method used, with reference to this part of ISO 14502;

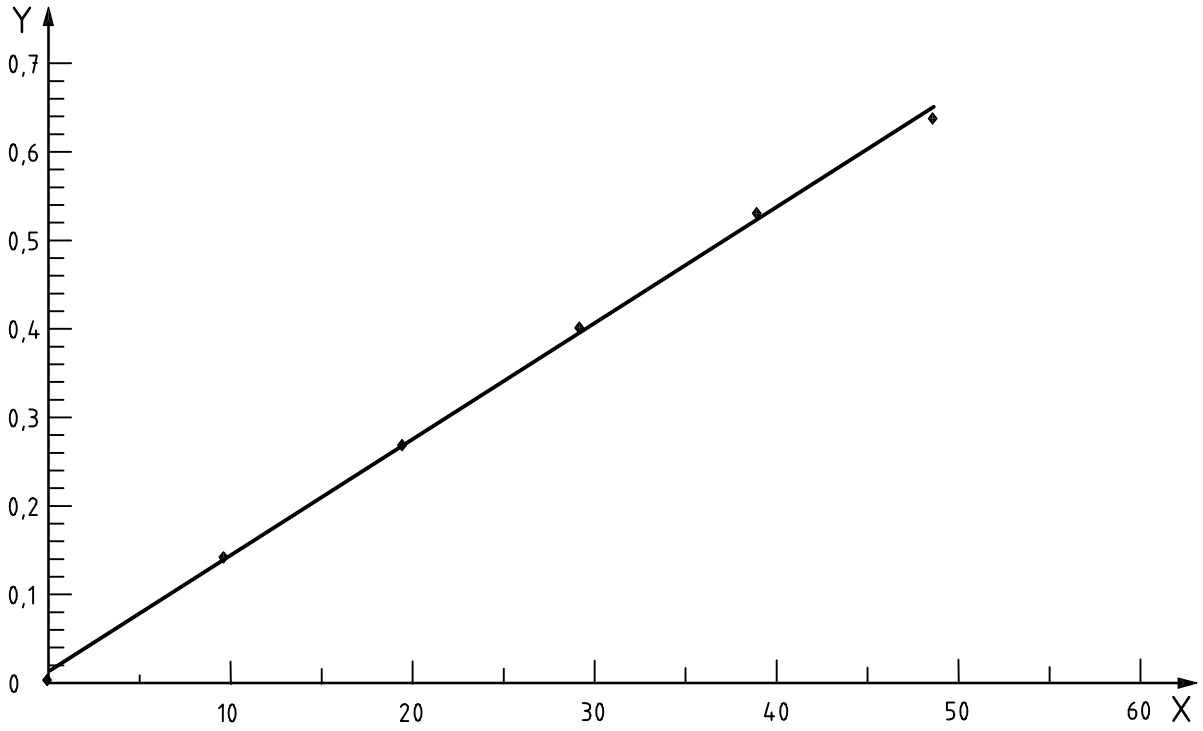
— all operating details not specified in this part of ISO 14502, or regarded as optional, together with details of any incidents which may have influenced the test result(s);

— the test result(s) obtained;

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

Annex A
(informative)

Gallic acid calibration graph



X Content of anhydrous gallic acid, µg/ml

Y Optical density, 765 nm

$y = 0{,}132x = 0{,}0113$

$R^2 = 0{,}998\ 5$

Calibration line slope = 0,013 2

Intercept value = 0,011 3

Figure A.1 — Best-fit linear calibration graph for gallic acid

Annex B
(informative)

Results of interlaboratory test

An interlaboratory test, carried out in 2001 under the auspices of the International Organization for Standardization, gave the statistical results (evaluated in accordance with ISO 5725-2) shown in Table B.1.

Table B.1 — Precision data

Sample identification	Green leaf tea A	Black leaf tea B	Black leaf tea C
Number of participating laboratories	23	23	23
Number of accepted test results	20	20	20
Mean total polyphenol content, % (mass fraction), on a dry matter basis	24,35	18,81	13,95
Repeatability standard deviation, s_r	0,332	0,218	0,214
Repeatability coefficient of variation, %	1,36	1,16	1,53
Repeatability limit, r (= 2,8 s_r)	0,93	0,61	0,60
Reproducibility standard deviation, s_R	1,129	1,186	1,029
Reproducibility coefficient of variation, %	4,64	6,31	7,38
Reproducibility limit, R (= 2,8 s_R)	3,16	3,32	2,88

Bibliography

[1] ISO 1839:1980, *Tea — Sampling*

[2] ISO 7516:1984, *Instant tea in solid form — Sampling*

[3] ISO 5725-2:1994, *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] SINGLETON, V.L., ORTHOFER, R. and LAMUELA-RAVENTOS, R.M. *Analysis of total phenolics and other oxidation substrates and antioxidants by means of Folin-Ciocalteu reagent.* In: *Methods in Enzymology. Oxidants and Antioxidants*, Part A, Lester Packer, ed. (1999) **299**, pp. 152-178

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