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Determination of substances characteristic of green and black tea —

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

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

Partie 2: Dosage des catechins dans le thé vert — Méthode par chromatographie en phase liquide à haute performance

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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 14502-2 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*:

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— *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*

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Determination of substances characteristic of green and black tea —

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

1 Scope

This part of ISO 14502 specifies a high-performance liquid chromatographic (HPLC) method for the determination of the total catechin content of tea from the summation of the individual catechins. It is applicable to both leaf and instant green tea, and with precision limitations to black tea (see Annex A).

Gallic acid and caffeine can also be determined by this method, as can theogallin and theaflavins.

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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.

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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

The total catechin content from a test portion of finely ground leaf tea is extracted with 70 % methanol at 70 °C. Instant teas are dissolved in hot water with 10 % (volume fraction) acetonitrile added to stabilize the extract. The individual catechins in the extract are determined by HPLC on a phenyl-bonded column using gradient elution with UV detection at 278 nm. External standards are used for quantitation. External catechin standards of defined purity and moisture content may be used directly. Alternatively, caffeine may be used as a standard in conjunction with individual catechin Relative Response Factors (RRFs) established by an ISO international interlaboratory test.

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, HPLC grade.

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4.3 Methanol.

4.4 Acetic acid, glacial HPLC grade.

4.5 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.

4.6 EDTA solution, 10 mg/ml.

Weigh (1,00 ± 0,01) g of EDTA (ethylenediaminetetraacetic acid disodium salt, dihydrate) into a 100 ml one-mark volumetric flask. Add sufficient warm water to half-fill the flask. Swirl to dissolve the EDTA, cool to room temperature, dilute to the mark with water and mix.

Prepare a fresh solution daily.

4.7 Ascorbic acid solution, 10 mg/ml.

Weigh (1,00 ± 0,01) g of L-ascorbic acid into a 100 ml one-mark volumetric flask. Dissolve in water, dilute to the mark and mix.

Prepare a fresh solution daily.

4.8 Stabilizing solution, 10 % (volume fraction) acetonitrile with 500 µg/ml of EDTA and ascorbic acid.

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Using a pipette, transfer 25 ml of EDTA solution (4.6), 25 ml ascorbic acid solution (4.7) and 50 ml of acetonitrile (4.2) to a 500 ml one-mark volumetric flask. Dilute to the mark with water and mix.

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Prepare a fresh solution daily.

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4.9 HPLC mobile phases.

SAFETY PRECAUTIONS — Wear gloves, eye protection and dispense reagents in a fume cupboard.

4.9.1 Mobile phase A, 9 % (volume fraction) acetonitrile, 2 % (volume fraction) acetic acid with 20 µg/ml EDTA.

Transfer 180 ml of acetonitrile (4.2) and 40 ml of acetic acid (4.4) to a 2 litre one-mark volumetric flask. Add sufficient water to half-fill the flask and add 4,0 ml of EDTA solution (4.6). Dilute to the mark with water, mix and filter through a filter of 0,45 µm pore size (5.10).

4.9.2 Mobile phase B, 80 % (volume fraction) acetonitrile, 2 % (volume fraction) acetic acid with 20 µg/ml EDTA.

Transfer 800 ml of acetonitrile (4.2) and 20 ml of acetic acid (4.4) into a 1 litre one-mark volumetric flask. Add approximately 100 ml water and 2,0 ml of EDTA solution (4.6). Dilute to the mark with water, mix and filter through a filter of 0,45 µm pore size (5.10).

4.10 Stock standard solutions.

4.10.1 General.

If catechins of known purity are available, they may be used directly as external standards. In addition to the normally quoted HPLC purity, it is important that their moisture contents be also known, as high levels of water of crystallization will not be accounted for in the HPLC assessment. The purity and moisture content data on standards used in the interlaboratory test are given in Annex B. If comprehensive purity data are unavailable or cannot be determined, catechin materials should only be used as marker compounds to aid identification. In these circumstances, quantitation may be achieved using a caffeine external standard in conjunction with

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consensus individual catechin RRF values (with respect to caffeine) obtained from interlaboratory tests (see 9.2 and Reference [3]).

4.10.2 Caffeine stock standard solution, corresponding to 2,00 mg/ml.

Weigh (0,200 ± 0,001) g of anhydrous caffeine into a 100 ml one-mark volumetric flask. Add sufficient warm water to half-fill the flask. Swirl to dissolve the caffeine then cool to room temperature. Dilute to the mark with water and mix.

4.10.3 Gallic acid stock standard solution, corresponding to approximately 1,00 mg/ml of anhydrous gallic acid.

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

Prepare fresh standard solution daily.

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 set by the American Chemical Society. If not known, the moisture content (loss in mass at 103 °C) should be determined on a portion of the standard material.

4.10.4 Preparation of individual catechin stock standard solutions

Accurately weigh the amounts of standards given in Table 1 into separate one-mark volumetric flasks. Dissolve in stabilizing solution (4.8), gently warming if necessary (max. 40 °C). Cool to room temperature, dilute to the mark with stabilizing solution and mix.

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Table 1 — Catechin stock standard solutions

Standard component	Mass of standard g	Volume of stabilizing solution ml	Nominal concentration of stock standard mg/ml
(+)-Catechin	0,020 ± 0,001	20	1,0
(–)-Epicatechin	0,020 ± 0,001	20	1,0
(–)-Epigallocatechin	0,040 ± 0,001	20	2,0
(–)-Epigallocatechin gallate	0,040 ± 0,001	20	2,0
(–)-Epicatechin gallate	0,040 ± 0,001	20	2,0

Where sufficient quantities (i.e. > 20 mg) are available, an analytical balance capable of weighing to an accuracy of at least 0,1 mg is required for the preparation of the individual stock standard solutions. For limited quantities (i.e. < 20 mg), an analytical balance capable of weighing to 0,01 mg is required.

4.11 Dilute standard solutions.

4.11.1 Dilute gallic acid standard solution, corresponding to approximately 200 µg/ml.

Using a pipette, transfer 20 ml of the gallic acid stock standard solution (4.10.3) to a 100 ml one-mark volumetric flask. Dilute to the mark with stabilizing solution (4.8) and mix.

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4.11.2 Mixed standard solutions.

Prepare the three mixed standard solutions A, B and C containing caffeine, gallic acid and the catechins being used for external standardization or as marker compounds. Carefully pipette the volumes given in Table 2 of caffeine stock standard solution (4.10.2), dilute gallic acid standard solution (4.11.1) and any available individual catechin stock standard solutions (4.10.4) into three separate 20 ml one-mark volumetric flasks. Dilute to the mark with stabilizing solution (4.8) and mix. Pipette 1,0 ml aliquots of each mixed standard solution into labelled small amber glass vials. Gently flush with nitrogen prior to sealing and store frozen at –20 °C. The nominal concentrations of components of standard solutions A, B and C are given in Table 3.

With catechins of unknown purity it is essential that an individual HPLC assessment be first carried out to check for other potentially interfering components.

NOTE The nominal concentrations of the mixed standard solutions A, B and C are given in Table 2 and have been selected to cover the range typically found in tea.

Calculate the actual anhydrous concentrations from the masses used for preparation of the stock standard solutions along with the standard moisture contents.

The mixed working standard solutions A, B and C will remain stable for at least 2 months when stored frozen at –20 °C. Only thaw sufficient mixed working standard solution vials for each batch of analysis. Discard any remaining solution, and do not refreeze.

Table 2 — Preparation of mixed standard solutions A, B and C

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Component	Solution	Aliquots required for the preparation of 20 ml of mixed standard solution ml		
		A	B	C
Caffeine	2,00 mg/ml caffeine stock standard solution (4.10.2)	0,5	1,0	1,5
Gallic acid	200 µg/ml dilute gallic acid standard solution (4.11.1)	0,5	1,0	2,5
+C	1,00 mg/ml +C stock standard solution (4.10.4)	1,0	2,0	3,0
EC	1,00 mg/ml EC stock standard solution (4.10.4)	1,0	2,0	3,0
EGC	2,00 mg/ml EGC stock standard solution (4.10.4)	1,0	2,0	3,0
EGCG	2,00 mg/ml EGCG stock standard solution (4.10.4)	1,0	2,0	4,0
ECG	2,00 mg/ml ECG stock standard solution (4.10.4)	0,5	1,0	2,0

Table 3 — Nominal concentrations of mixed standard solutions A, B and C

Component	Nominal concentration µg/ml		
	A	B	C
Gallic acid	5	10	25
Caffeine	50	100	150
+C	50	100	150
EC	50	100	150
EGC	100	200	300
EGCG	100	200	400
ECG	50	100	200

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5 Apparatus

Usual laboratory apparatus and, in particular, the following.

5.1 Analytical balances, capable of weighing to an accuracy of ± 0,000 1 g and ± 0,000 01 g (see 4.10.4).

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

5.3 Dispenser, for methanol/water extraction mixture (4.5), 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 Vortex mixer, for efficient mixing during extraction.

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

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

5.8 One-mark volumetric flasks, of capacities 5 ml, 10 ml, 20 ml, 100 ml, 500 ml, 1 litre and 2 litres.

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

5.10 Filters, membrane filter units of 0,45 µm pore size for filtration of mobile phases and diluted sample extracts.

NOTE PTFE and nylon membrane filters have proven to be suitable.

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All membranes should be checked to ensure that that catechin retention does not occur.

5.11 High-performance liquid chromatograph, equipped to perform binary gradient elution, with a thermostatically controlled column compartment and an ultraviolet detector set at 278 nm.

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5.12 Data collection /integration system.

5.13 Chromatographic column for HPLC.

NOTE Phenyl-bonded phases give additional selectivity over reversed-phase materials, and result in improved resolution of the catechins. In this part of ISO 14502 the chromatographic conditions and composition of the mobile phase specified (4.9) are suitable for a Phenomenex Luna 5 µm Phenyl-Hexyl®[1] column of dimensions 250 mm × 4,6 mm, fitted with a Phenomenex SecurityGuard®[2] 4 mm × 3,0 mm Phenyl-Hexyl cartridge. If other types of column are used, modifications to the mobile phase and chromatographic conditions may be 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.

1) Phenyl-Hexyl® is an example of a suitable product available commercially. This information is given for the convenience of users of this part of ISO 14502 and does not constitute an endorsement by ISO of this product.

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7 Preparation of test samples

To ensure homogeniety, 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 (e.g. freeze-dried instant tea).

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

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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.

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8.3.2 Leaf tea

Weigh (0,200 ± 0,001) g of the test sample (Clause 7) into an extraction tube (5.6).

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 it 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 (4.5) 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 (5.5).

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.

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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 (5.7) or a 10 ml one-mark volumetric flask (5.8).

8.4.2.6 Repeat extraction steps 8.4.2.2 to 8.4.2.5. Combine the two extracts, make up to 10 ml with cold methanol/water extraction mixture (4.5) 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) to a graduated tube (5.7). Dilute to 5,0 ml with stabilizing solution (4.8). Mix and filter through a 0,45 µm filter (5.10).

8.6 Determination

8.6.1 General

Catechins are very susceptible to degradation, and metal ion contamination of the chromatographic system appears to be a major contributing factor (see Annex D). Although addition of EDTA to the mobile phase can help minimize these effects, it is important to maintain a clean chromatographic system. It is recommended to thoroughly flush the system with, for example, 50 % (volume fraction) acetonitrile (or initially an appropriate miscible solvent depending on previous application) before and after use to remove residual buffer salts and acids, and to prevent corrosion.

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8.6.2 Adjustment of the apparatus

Set up the chromatograph (5.11) in accordance with the manufacturer's instructions and adjust it as follows.

a) Flow rate of the mobile phase (4.9): 1,0 ml.

b) Binary gradient conditions: 100 % mobile phase A (4.9.1) for 10 min, then over 15 min a linear gradient to 68 % mobile phase A, 32 % mobile phase B (4.9.2) and hold at this composition for 10 min. Then reset to 100 % mobile phase A and allow to equilibrate for 10 min before next injection.

c) Temperature of the column (5.13): 35 °C ± 0,5 °C. Column temperature control is essential (chromatography column oven or recirculating water jacket) if major drifts in retention times are to be avoided.

d) UV detector set at 278 nm. Ensure that the detector sensitivity range selected is able to keep all peaks from the highest mixed working standard C within the scale of the data collection system used.

8.6.3 HPLC analysis

Once the flow rate of the mobile phase (4.9) and temperature are stable, condition the column with a blank gradient run (8.6.2). Then inject onto the column 10 µl of each of the mixed standard solutions A, B and C (4.11.2), followed by an equal volume of the diluted sample extract (8.5). Repeat the injection of the mixed working standard solutions at regular intervals (typically after six test solutions). Collect data using the data collection/integration system (5.12) for all peaks in the mixed standards and test sample solutions.

After each batch of analysis, thoroughly flush the chromatographic system and column with 50 % (volume fraction) acetonitrile (see 8.6.1) and replace column sealing plugs if disconnected for storage.

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