INTERNATIONAL STANDARD

ISO 14502-2

First edition
2005-03-01

Determination of substances characteristic of green and black tea —

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

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

Reference number
ISO 14502-2:2005(E)

ISO copyright office
Case postale 56 • CH-1211 Geneva 20
Tel. + 41 22 749 01 11
Fax + 41 22 749 09 47
E-mail copyright@iso.org
Web www.iso.org

Published in Switzerland

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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*:

— *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 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.

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

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.

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.

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.

Prepare a fresh solution daily.

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

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.

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.

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

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

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.

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.

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.

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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.

2) SecurityGuard® 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.

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

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 (5.6).

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.

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.

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.

8.6.4 Identification

Identify the individual catechins by comparing retention times from sample chromatograms with those obtained from the mixed standard solutions obtained under the same chromatographic conditions (8.6.2). The use of diode array detection allows the UV profile of the catechin peaks to be scrutinized and peak purity assessed, which can be particularly useful for the determination of the low levels of catechins in black tea.

NOTE Where the availability of catechin marker compounds is limited, analysis of a green leaf tea and comparison with a typical HPLC chromatogram as shown in Annex C will aid identification.

9 Calculation

9.1 General

Quantitation is performed by external standardization, using

— either individual catechin standards of established purity and moisture content, or

— a caffeine standard used in conjunction with consensus individual catechin RRFs measured with respect to caffeine (see 4.10.1 and 9.2).

9.2 Quantitation using catechin standards

9.2.1 Calculate to the nearest 0,1 g/ml the concentration of anhydrous standard in each of the mixed standard solutions A, B and C (4.11.2).

9.2.2 Construct linear calibration graphs for each standard from the anhydrous concentrations (μg/ml) against the peak areas obtained from the data collection/integration system (5.12) and obtain the slope and intercept values.

9.2.3 The individual component content, w_C, expressed as a percentage by mass on a sample dry matter basis, is given by the formula:

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

where

A_{sample} is the peak area of the individual component in the test sample;

$A_{intercept}$ is the peak area at the point the standard calibration line intercepts the y-axis;

S_{std} is the standard calibration line slope;

F_{std} is the Relative Response Factor, measured with respect to caffeine for the individual component;

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 (see 8.5), typically 5;

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

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

9.2.4 The total catechin content, w_T, expressed as a mass fraction in percent on a sample dry matter basis, is given by the summation of the individual catechins (see Table 4 for abbreviations):

w_T = (% EGC) + (% +C) + (% EC) + (% EGCG) + (% ECG)

9.3 Quantitation using a caffeine standard and catechin Relative Response Factors (RRFs)

9.3.1 The RRF values (measured with respect to caffeine) for gallic acid and the individual catechins obtained from the international interlaboratory test[3] are given in Table 4. These consensus values, obtained on standards of known purity and expressed on an anhydrous standard basis, enable quantitation to be achieved with reference to the caffeine standard. Comparison of results obtained using either catechin standards or a caffeine standard with catechin RRFs is given in Annex E.

Table 4 — Consensus Relative Response Factors

Component	**Relative Response Factor (RRF) with respect to caffeine** (calculated on standard dry matter basis)
Gallic acid	0,84
(−)-Epigallocatechin (EGC)	11,24
(+)-Catechin (+C)	3,58
(−)-Epicatechin (EC)	3,67
(−)-Epigallocatechin gallate (EGCG)	1,72
(−)-Epicatechin gallate (ECG)	1,42

9.3.2 Construct a linear caffeine calibration graph from the anhydrous concentrations (µg/ml) against the caffeine peak areas obtained for each of the mixed standard solutions A, B and C (4.11.2) and obtain the slope and intercept value.

9.3.3 The identified individual component (see 8.6.4) content, expressed as a percentage by mass on a sample dry matter basis, is given by the formula:

$$w_C = \frac{(A_{sample} - A_{intercept}) \times F_{std} \times V_{sample} \times d \times 100}{S_{caffeine} \times m_{sample} \times 10\,000 \times w_{DM,sample}}$$

where

A_{sample} is the peak area of the individual component in the test sample;

$A_{intercept}$ is the peak area at the point the caffeine calibration line intercepts the y-axis;

$S_{caffeine}$ is the caffeine calibration line slope;

F_{std} is the Relative Response Factor, measured with respect to caffeine for the individual component;

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 (see 8.5), typically 5;

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

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

9.3.4 The total catechin content as a percentage by mass on a sample dry matter basis, is given by the summation of the individual catechins (see Table 4 for abbreviations):

$$w_T = (\%\ EGC) + (\%\ +C) + (\%\ EC) + (\%\ EGCG) + (\%\ ECG)$$

10 Precision

10.1 Interlaboratory test

Details of the interlaboratory test to determine the precision of the method are summarized in Annex A. 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 (r) values given in Table A.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 (R) values given in Table A.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)

Results of interlaboratory tests

Interlaboratory tests, carried out during 1999/2002 under the auspices of the International Organization for Standardization, gave the statistical results (evaluated in accordance with ISO 5725-2) shown in Tables A.1 and A.2.

NOTE Details are given in Reference [3].

The results in both Tables A.1 and A.2. were obtained from quantitation using a caffeine standard in conjunction with consensus individual catechin Relative Response Factors (RRFs). The results shown in Table A.1 were obtained prior to the recommendation to incorporate EDTA into the mobile phase (see Annex D), whereas the results in Table A.2 were obtained after EDTA had been included in the mobile phase. Total catechin content is expressed in percent, on an anhydrous standard and sample dry matter basis.

Table A.1 — Precision data (no EDTA)

Sample identification	Sample 1 Green leaf tea	Sample 2 Green leaf tea	Sample 3 Black leaf tea	Sample 4 Black leaf tea
Number of participating laboratories	14	14	14	14
Number of accepted test results	9	10	11	10
Mean total catechin content, % (mass fraction), on dry matter basis	12,30	15,70	9,53	7,19
Repeatability standard deviation, s_r	0,194	0,163	0,221	0,095
Repeatability coefficient of variation, %	1,58	1,04	2,32	1,32
Repeatability limit, r (= 2,8 s_r)	0,54	0,46	0,62	0,27
Reproducibility standard deviation, s_R	0,888	1,664	1,066	0,925
Reproducibility coefficient of variation, %	7,22	10,60	11,19	12,87
Reproducibility limit, R (= 2,8 s_R)	2,49	4,66	2,98	2,59

Table A.2 — Precision data – EDTA included in mobile phase

Sample identification	Sample 1 Green leaf tea	Sample 2 Green leaf tea	Sample 3 Black leaf tea	Sample 4 Black leaf tea
Number of participating laboratories	11	11	11	11
Number of accepted test results	9	9	9	9
Mean total catechin content, % (mass fraction), on dry matter basis	12,14	14,78	8,93	6,81
Repeatability standard deviation, s_r	0,21	0,43	0,17	0,19
Repeatability coefficient of variation, %	1,75	2,93	1,87	2,75
Repeatability limit, r (= 2,8 s_r)	0,59	1,21	0,47	0,52
Reproducibility standard deviation, s_R	1,21	1,33	0,67	0,58
Reproducibility coefficient of variation, %	10,00	8,97	7,52	8,48
Reproducibility limit, R (= 2,8 s_R)	3,40	3,71	1,88	1,62

NOTE The black leaf teas, samples 3 and 4, are both lightly fermented Darjeeling, and therefore still have a reasonable catechin content. More extensively fermented black teas will have lower catechin contents, and would therefore be expected to have a negative effect on the precision data.

Annex B
(informative)

Assessment of purity of standards

Comprehensive purity assessments, in addition to HPLC peak purity checks, were carried out on the batches of standard materials used in the interlaboratory test. The purity data obtained are given in Table B.1.

Table B.1 — Standard purity data

Standard	Source	Supplied purity information	% Purity HPLC[a] (Determined)	% Purity NMR[b] (Determined)	% Moisture KF (Determined)	% Moisture (Theoretical formula values for comparison)	
Gallic acid	Sigma	Crystalline, anhydrous	100	—	1,7[c]	Anhydrous $1H_2O$	0 9,6
+C	Sigma	Min. 98 % $2H_2O$	99	92	8,0	$1H_2O$ $2H_2O$	5,8 11,0
EC	Sigma	—	98	96	1,8	$1H_2O$	5,8
EGC	Isolated[d]	Approx. 96 %	96	88	18,8	$4H_2O$	19,0
EGCG	Isolated[d]	Approx. 95 %	99	91	14,4	$4H_2O$	13,6
ECG	Isolated[d]	Approx. 97 %	99	89	13,4	$4H_2O$	14,0

[a] HPLC purity is the polyphenol peak area as a % of total area of all peaks in chromatogram.

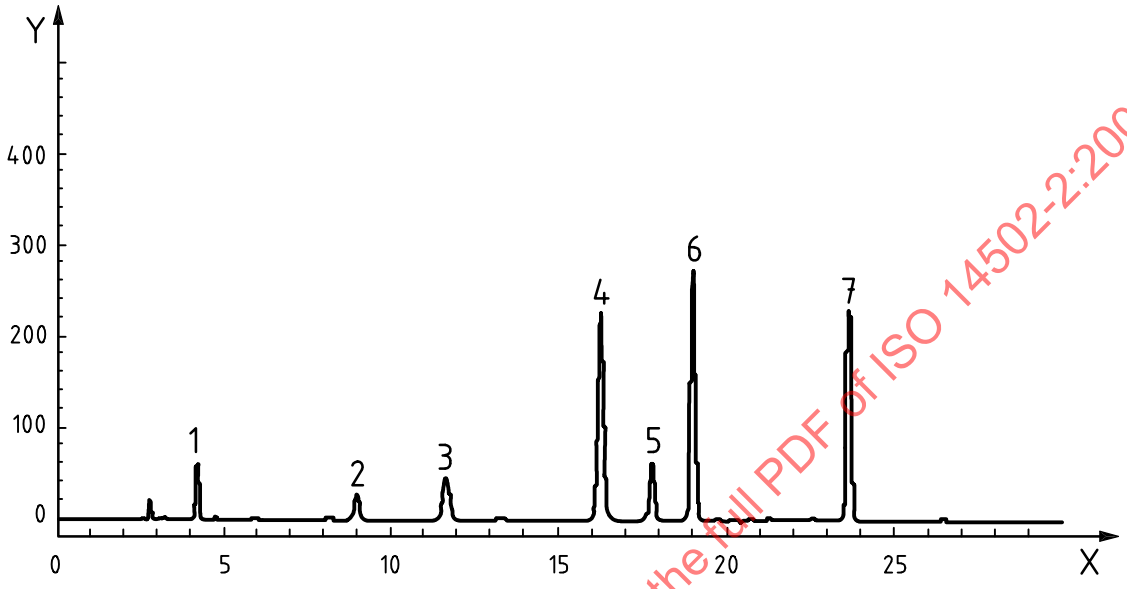
[b] NMR purity obtained by proton counting against a caffeine standard.

[c] Moisture by Karl-Fischer titration except gallic acid which was determined by oven drying (loss of mass at 103 °C).

[d] Isolated from green tea water solubles by ethyl acetate extraction, separation by preparative gel permeation, and purification by preparative HPLC and recrystallization.

Annex C
(informative)

Typical HPLC chromatograms

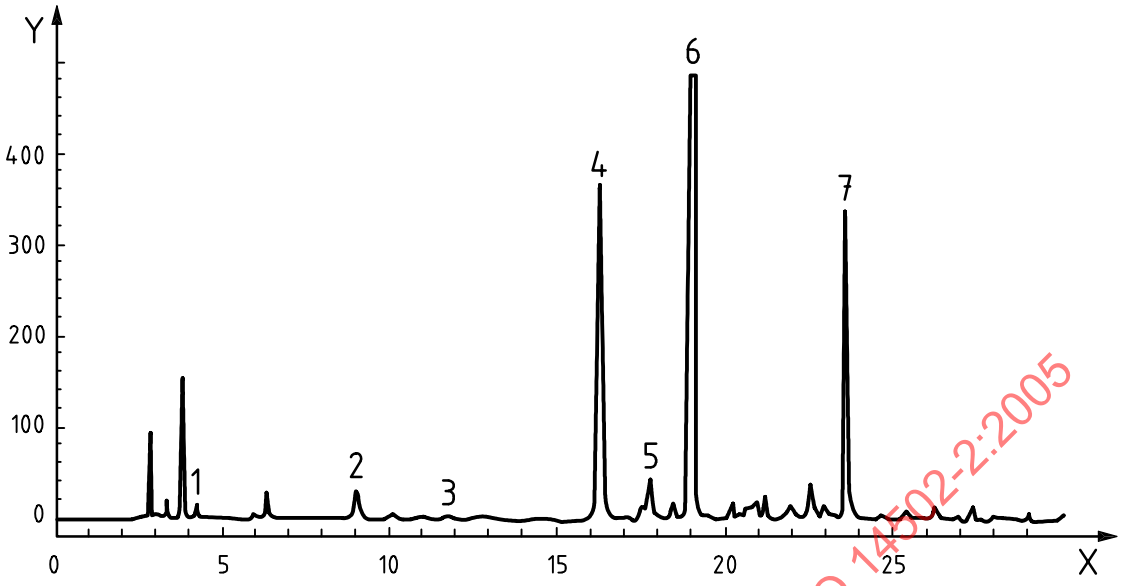


Key

X Retention time, min
Y Response, mAU

1 gallic acid
2 EGC
3 catechin
4 caffeine
5 epicatechin
6 EGCG
7 ECG

Figure C.1 — Mixed catechin standard B



Key

X Retention time, min

Y Response, mAU

1 gallic acid

2 EGC

3 catechin

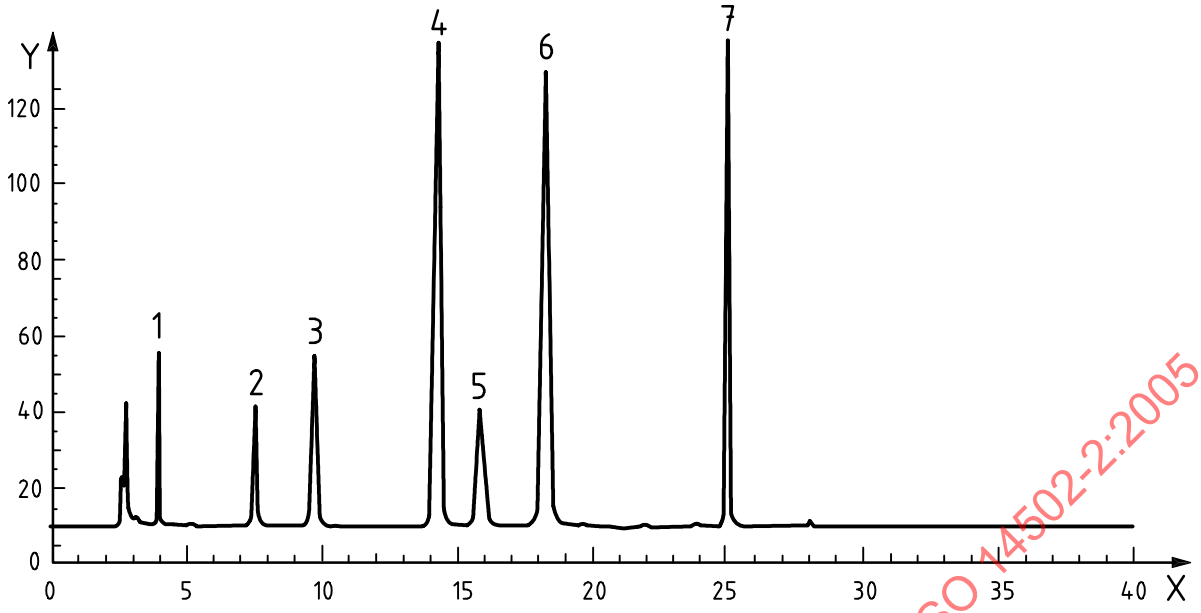
4 caffeine

5 epicatechin

6 EGCG

7 ECG

Figure C.2 — Green leaf tea extract



Key

X Retention time, min

Y Response, mAU

1 gallic acid

2 EGC

3 catechin

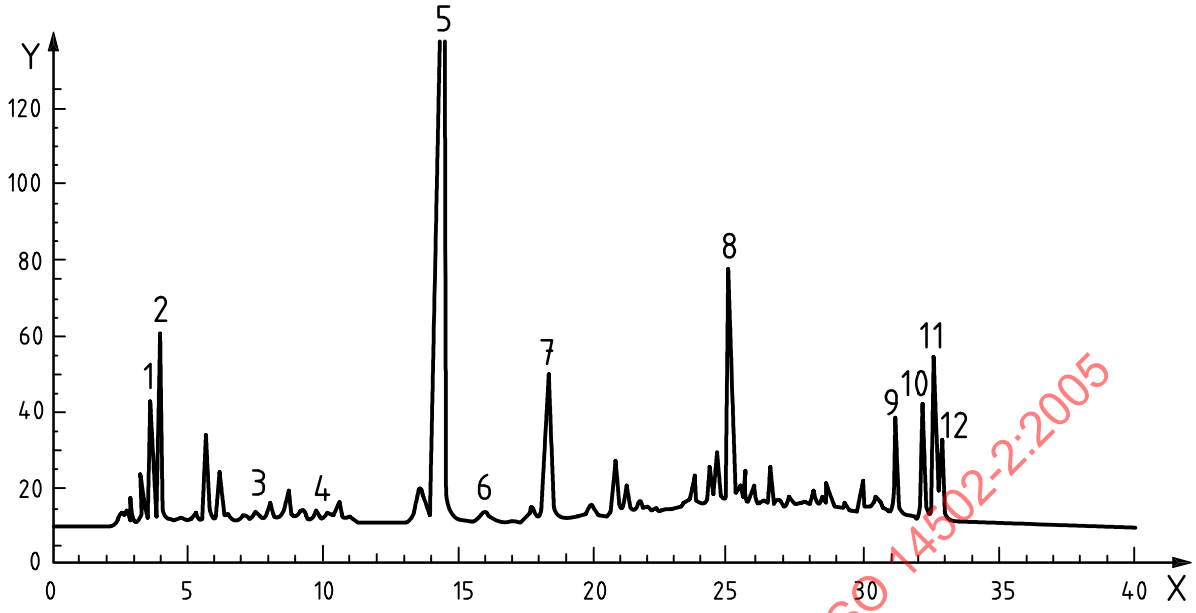
4 caffeine

5 epicatechin

6 EGCG

7 ECG

Figure C.3 — Mixed catechin standard



Key

X Retention time, min

Y Response, mAU

1 theogallin
2 gallic acid
3 EGC
4 catechin
5 caffeine
6 epicatechin
7 EGCG
8 ECG
9 TF
10 TFDG
11 TF-3-MG
12 TF-3-MG

Figure C.4 — Black leaf tea extract

Annex D
(informative)

The effect of ferric ions on catechin RRFs

The data obtained from this investigation, shown in Tables D.1 and Figures D.1 and D.2, show that:

a) low levels of Fe^{3+} ions can cause significant on-column degradation of the catechins;

b) EGCG and EGC are considerably more susceptible to degradation;

c) the addition of 20 µg/ml EDTA to the mobile phase effectively sequesters the Fe^{3+} ions and prevents catechin degradation;

d) excellent agreement (within 5 %) was obtained with the consensus RRFs after the addition of EDTA;

e) the EDTA already present in the mixed catechin standard (from stabilizing solution) does not provide protection against a contaminated chromatography system;

f) it is important to maintain a clean chromatographic system, but inclusion of EDTA in the mobile phase will help improve the robustness of the method.

Table D.1 — Effect of Fe^{3+} on catechin RRFs

Component	Relative Response Factors (RRFs) with respect to caffeine (Calculated on standard dry matter basis)[a]				
	Consensus RRFs	After addition of 0,1 µg/ml Fe^{3+}	After addition of 0,5 µg/ml Fe^{3+}	After addition of 0,5 µg/ml Fe^{3+} and 20 g/ml EDTA	After addition of 20 µg/ml EDTA
Gallic acid	0,84	0,84 [0]	1,03 [19]	0,81 [0]	0,81 [0]
EGC	11,24	16,38 [31]	24,64 [54]	11,79 [5]	11,79 [5]
+C	3,58	3,76 [5]	3,85 [7]	3,58 [0]	3,65 [2]
EC	3,67	3,99 [8]	3,92 [6]	3,75 [2]	3,86 [5]
EGCG	1,72	3,11 [45]	5,80 [70]	1,72 [0]	1,73 [0]
ECG	1,42	1,54 [8]	1,73 [18]	1,42 [0]	1,43 [0]
[a] % reduction in response compared to consensus RRFs given in [ ].					
NOTE $FeCl_3$ used for the source of Fe^{3+} ions.					