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## Tea — Determination of theaflavins in black tea — Method using high performance liquid chromatography

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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.

The procedures used to develop this document and those intended for its further maintenance are described in the ISO/IEC Directives, Part 1. In particular, the different approval criteria needed for the different types of ISO documents should be noted. This document was drafted in accordance with the editorial rules of the ISO/IEC Directives, Part 2 (see [www.iso.org/directives](http://www.iso.org/directives)).

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Any trade name used in this document is information given for the convenience of users and does not constitute an endorsement.

For an explanation of the voluntary nature of standards, the meaning of ISO specific terms and expressions related to conformity assessment, as well as information about ISO's adherence to the World Trade Organization (WTO) principles in the Technical Barriers to Trade (TBT), see [www.iso.org/iso/foreword.html](http://www.iso.org/iso/foreword.html).

This document was prepared by Technical Committee ISO/TC 34, *Food products*, Subcommittee SC 8, *Tea*.

Any feedback or questions on this document should be directed to the user's national standards body. A complete listing of these bodies can be found at [www.iso.org/members.html](http://www.iso.org/members.html).

# Tea — Determination of theaflavins in black tea — Method using high performance liquid chromatography

## 1 Scope

This document specifies a high performance liquid chromatography (HPLC) or ultra-high performance liquid chromatography (UHPLC) method for the determination of content of the four major theaflavins of tea.

It is applicable to both leaf and instant black and oolong teas. The method is currently not validated for ready-to-drink (RTD) beverages.

## 2 Normative references

The following documents are referred to in the text in such a way that some or all of their content constitutes requirements 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, *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 Terms and definitions

No terms and definitions are listed in this document.

ISO and IEC maintain terminological databases for use in standardization at the following addresses:

- ISO Online browsing platform: available at <https://www.iso.org/obp>
- IEC Electropedia: available at <http://www.electropedia.org/>

## 4 Principle

Extraction of the theaflavins from a test portion of finely ground leaf tea is achieved with 70 % methanol at 70 °C. Instant teas are dissolved in hot water with a volume fraction of 10 % acetonitrile added to stabilize the extract. The individual theaflavins in the extract are determined by HPLC on a reversed-phase column using isocratic elution with UV detection at 274 nm, optional at 375 nm (as an alternative detection wavelength not used in the method validation). External standards are used for quantitation. External theaflavin standards of defined purity and moisture content may be used directly. Alternatively, caffeine may be used as a standard in conjunction with individual theaflavins relative response factors (RRFs) established by an ISO international interlaboratory test (see [Table 3](#)).

## 5 Reagents

### 5.1 General

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

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

## ISO/FDIS 18447:2021(E)

**5.1.1 Water**, grade 1 in accordance with ISO 3696.

**5.1.2 Acetonitrile**, HPLC grade.

**5.1.3 Methanol**, HPLC grade.

**5.1.4 Acetic acid**, glacial HPLC grade.

**5.1.5 Ascorbic acid**, analytical grade.

**5.1.6 Ethylenediaminetetraacetic acid disodium salt, dihydrate (EDTA)**, analytical grade.

**5.1.7 Methanol/water extraction mixture**, a volume fraction of 70 % methanol.

Add 700 ml of the methanol (5.1.3) to a 1 l one-mark volumetric flask. Dilute to the mark with water (5.1.1) and mix.

**5.1.8 20 % acetonitrile stabilizer solution.**

Fill a 500 ml volumetric flask half with water (5.1.1). Add 10 ml of glacial acetic acid (5.1.4) and 100 ml of acetonitrile (5.1.2). Add 125 mg each of ascorbic acid (5.1.5) and EDTA (5.1.6) and fill up to the mark with water (5.1.1). This solution may be stored in the fridge for up to a week.

**5.1.9 Leaf/infusion stabilizing solution.**

Weigh 0,062 5 g EDTA (5.1.6) and 0,062 5 g ascorbic acid (5.1.5) into a 25 ml volumetric flask and fill up to the mark with water (5.1.1), giving a solution containing 2,5 mg/ml EDTA and 2,5 mg/ml ascorbic acid. This solution may be stored in the fridge for up to a week.

**5.2 HPLC mobile phases**

**5.2.1 Mobile phase A**, 2 % (volume fraction) acetic acid in water.

Transfer 40 ml of acetic acid (5.1.4) into a 2 l one-mark volumetric flask. Add sufficient water (5.1.1) to half fill the flask and mix well. Dilute to the mark with water, mix and filter (6.10).

**5.2.2 Mobile phase B**, 2 % (volume fraction) acetic acid in acetonitrile.

Transfer 20 ml of acetic acid (5.1.4) into a 1 l one-mark volumetric flask. Add approximately 400 ml acetonitrile (5.1.2), mix well and dilute to the mark with acetonitrile, mix again and filter.

**5.3 Stock standard solutions**

**5.3.1 General**

If theaflavins of known and guaranteed 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 are also known, as high levels of water of crystallization will not be accounted for in the HPLC assessment. Purity and moisture content data on standards used in ISO interlaboratory testing are given in Annex A. If comprehensive purity data are unavailable or cannot be determined, theaflavins should only be used as marker compounds to aid identification. In these circumstances, quantification may be achieved using a caffeine external standard in conjunction with consensus individual theaflavin RRF values (with respect to caffeine) obtained from ISO interlaboratory testing (see Table 3).

NOTE The statistical data in Annex A have been generated using the calibration against caffeine (see 5.5).

### 5.3.2 Preparation of individual theaflavin stock standard solutions

Weigh approximately 20 mg (exact weight recorded) of the following individual theaflavins into separate 10 ml volumetric flasks: theaflavin (TF), theaflavin-3-gallate (TF-3-g), theaflavin-3'-gallate (TF-3'-g) and theaflavin-3,3'-gallate (TF-3,3'-dig). Fill up to mark with 20 % acetonitrile stabilizing solution (5.1.8) to give stock standards with a concentration of approximately 2 mg/ml.

NOTE 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, whereas for limited quantities (i.e. < 20 mg) an analytical balance capable of weighing to 0,01 mg is required.

### 5.4 Mixed standard solutions

To prepare the mixed standards, pipette the aliquots of the individual standards into 20 ml volumetric flasks in accordance with Table 1 and fill up to the mark with 20 % acetonitrile stabilizing solution (5.1.8).

Table 1 — Preparation of mixed TF working strength standards

Standard name	Individual stock theaflavin standards diluted to 20 ml ml	Nominal concentration of individual theaflavins mg/l	Nominal concentration of injection volume µg/10 µl
A 4,0 µg std	4	400	4,0
B 3,0 µg std	3	300	3,0
C 2,0 µg std	2	200	2,0
D 1,0 µg std	1	100	1,0
E 0,5 µg std	0,5	50	0,5
F 0,25 µg std	0,25	25	0,25

The concentration of the individual TFs given in Table 1 is based on 100 % purity of the standards. The purity of the standard TFs should be determined by high resolution nuclear magnetic resonance (NMR) spectroscopy and the nominal concentrations of the standards accordingly should give the exact concentration of the individual TFs in each standard.

With theaflavins of unknown purity, it is strongly recommended that an individual HPLC assessment is first carried out to check for other potentially interfering components.

The nominal concentrations of the mixed standard solutions A to F are given in Table 1 and have been selected to cover the range typically found in tea. Calculate actual anhydrous concentrations from the weights used for preparation of the stock standard solutions along with the standard moisture contents.

The mixed working standard solutions A to F will remain stable for at least two 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.

### 5.5 Caffeine standard — Preparation of caffeine stock 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 to give a stock standard with a concentration of 2 mg/ml.

Prepare aliquots of the caffeine stock standard into 20 ml volumetric flasks as detailed in Table 2 and make up to volume with 20 % acetonitrile stabilizing solution (5.1.8).

Table 2 — Preparation of caffeine working strength standards

Standard name	Individual stock caffeine standards diluted to 20 ml ml	Nominal concentration of caffeine mg/l	Nominal concentration of injection volume $\mu\text{g}/10 \mu\text{l}$
<b>A</b> 1,0 $\mu\text{g}$ std	1	100	1,0
<b>B</b> 0,5 $\mu\text{g}$ std	0,5	50	0,5
<b>C</b> 0,25 $\mu\text{g}$ std	0,25	25	0,25
<b>D</b> 0,1 $\mu\text{g}$ std	0,1	10	0,1
<b>E</b> 0,05 $\mu\text{g}$ std	0,05	5	0,05
<b>F</b> 0,025 $\mu\text{g}$ std	0,025	2,5	0,025

NOTE Lower concentrations of standards (e.g. G corresponding to 1 mg/l are optional). Higher concentrations might yield a nonlinear calibration plot.

## 6 Apparatus

Use usual laboratory apparatus and, in particular, the following.

- 6.1 Analytical balances**, accuracy of  $\pm 0,000 1 \text{ g}$  and  $\pm 0,000 01 \text{ g}$  (See Note under [5.3.2](#)).
- 6.2 Water bath**, of  $(70 \pm 1) \text{ }^\circ\text{C}$ .
- 6.3 Dispenser**, for methanol/water extraction mixture ([5.1.5](#)), and set at 5,0 ml.
- 6.4 Centrifuge**, capable of 2 000 relative centrifugal force (typically 3 500 r/min).
- 6.5 Vortex mixer**, for efficient mixing during extraction.
- 6.6 Extraction tubes**, glass, 10 ml capacity, stoppered and able to withstand centrifugation.
- 6.7 Graduated tubes**, glass, 10 ml capacity with 0,1 ml graduations.
- 6.8 Volumetric flasks**, 5 ml, 10 ml, 20 ml, 100 ml, 500 ml, 1 l and 2 l one-mark flasks.
- 6.9 Pipettes**, glass or automatic, to cover the volume range for standard and sample extract dilutions.
- 6.10 Filters**, membrane filter units of  $0,45 \mu\text{m}$  and  $0,2 \mu\text{m}$  (for UHPLC) pore size for filtration of mobile phases and diluted sample extracts.
- PTFE and nylon membrane filters have proven to be suitable, but all membranes shall be checked to ensure that theaflavin retention does not occur.
- 6.11 High performance liquid chromatograph**, equipped to perform isocratic elution, with a thermostatically controlled column compartment and an ultraviolet detector set at 278 nm and (optional) at 375 nm.

The use of a photo-diode array detector is recommended. Alternatively, an ultra high performance liquid chromatograph can be used.

When using an autosampler, it is strongly recommended to use a device with a cooling possibility to ensure that sample degradation does not occur. Set at  $10 \text{ }^\circ\text{C}$ .



## 6.12 Data collection/integration system.

**6.13 Chromatographic column for HPLC.** Suitable RP columns for HPLC, dimensions e.g. 100 × 4,6, 150 × 4,6 or 250 × 4,6; i.d. of 2 mm is also possible (the flow has to be adjusted when using 2 mm columns, particle size 5 μ, 3,5 μ or 3 μ. For UPLC, typical dimensions are 100 mm × 2,1 mm, particle diameter is 1,8 μ.

NOTE Hypersil C18, 3 μ, 100 mm × 4,6 mm or a Zorbax Eclipse XBD 18, 3,5 μ, 150 mm × 4,6 mm fitted with a C18 security guard cartridge (e.g. Phenomenex) or similar<sup>1)</sup>. For UHPLC, use, for example, an Eclipse Plus C18 RRHD, 1,8 μ, 100 mm × 2,1 mm<sup>2)</sup>.

## 7 Sampling

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

Sampling is not part of the method specified in this document. A recommended sampling method is given in:

- ISO 1839 for leaf tea;
- ISO 7516 for instant tea.

## 8 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 (e.g. freeze-dried instant tea).

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## 9 Procedure

### 9.1 General

If it is required to check whether the repeatability requirement (11.2) is met, carry out two single determinations in accordance with 9.2 to 9.6 under repeatability conditions.

### 9.2 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 (see Clause 8) in accordance with:

- ISO 1572 for leaf tea;
- ISO 7513 for instant tea.

### 9.3 Test portion

#### 9.3.1 Instant tea

Weigh (0,2 ± 0,001) g of the test sample (see Clause 7) into a 10 ml one-mark volumetric flask.

1) These are examples of suitable products available commercially. This information is given for the convenience of users of this document and does not constitute an endorsement by ISO of these products. Equivalent products may be used if they can be shown to lead to the same results.

### 9.3.2 Leaf tea

Weigh  $(0,2 \pm 0,001)$  g of the test sample (see [Clause 7](#)) into an extraction tube ([6.6](#)).

## 9.4 Extraction of theaflavins

### 9.4.1 Instant tea

Add to the instant tea in the flask ([9.3.1](#)) 20 % acetonitrile stabilizing solution ([5.1.8](#)). Ensure that the sample has dissolved and sonicate if necessary. Fill up to the mark with 20 % acetonitrile stabilizing solution ([5.1.8](#)), mix and filter ([6.10](#)). For UHPLC, use a 0,2  $\mu\text{m}$  filter.

### 9.4.2 Leaf tea

**9.4.2.1** Place the methanol/water extraction mixture ([5.1.5](#)) contained in the dispenser ([6.3](#)) into the water bath ([6.2](#)) set at 70 °C, and allow at least 30 min for the extraction mixture to equilibrate.

**9.4.2.2** Place the extraction tube containing the sample ([9.3.2](#)) into the water bath set at 70 °C. Dispense 5,0 ml of hot methanol/water extraction mixture from [9.4.2.1](#) into the extraction tube, stopper and mix on the vortex mixer ([6.5](#)).

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

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

**9.4.2.4** Remove the extraction tube from the water bath and allow to cool to room temperature. Remove the stopper and place the tube in the centrifuge ([6.4](#)) at 3 500 r/min for 10 min.

**9.4.2.5** Carefully decant the supernatant into a graduated tube ([6.7](#)) or a 10 ml one-mark volumetric flask ([6.8](#)).

**9.4.2.6** Repeat extraction steps [9.4.2.2](#) to [9.4.2.5](#). Combine the two extracts, fill up to 10 ml with cold methanol/water extraction mixture ([5.1.5](#)) and mix the contents.

The extract from [9.4.2.6](#) 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 can settle during storage is not necessary.

## 9.5 Dilution

If necessary, use a pipette and transfer 1,0 ml of the sample extract (instant tea extract from [9.4.1](#) or leaf tea extract from [9.4.2.6](#)) into a graduated tube ([6.7](#)) and dilute to 2,0 to 5,0 ml with stabilizing solution ([5.1.9](#)). In case of leaf tea extracts, a degradation can occur on prolonged storage time prior to analysis if no stabilization solution is used. Mix and filter ([6.10](#)). For UHPLC, use a 0,2  $\mu\text{m}$  filter.

## 9.6 Determination

### 9.6.1 General

Theaflavins are very susceptible to degradation, and metal ion contamination of the chromatographic system appears to be a major contributing factor. Thoroughly flushing the system, e.g. with a volume fraction of 50 % 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 is recommended.

### 9.6.2 Adjustment of the apparatus

Set up the chromatograph (6.11) in accordance with the manufacturer's instructions and adjust it as follows.

- a) Flow rate of the mobile phase (5.2): 2,0 ml/min (for UHPLC: 0,5 ml to 1 ml/min), both depending on the column used.
- b) Conditions: 78 % mobile phase A (5.2.1) and 22 % mobile phase B (5.2.2); depending on the column used it might be necessary to adjust the solvent composition slightly (e.g. 77/23 or 79/21).
- c) Run time: 25 min (for UHPLC: 8 min to 10 min), both depending on the column used and the solvent composition.
- d) Temperature of the column (6.13): 30 °C ± 0,5 °C.
- e) When using a thermostatted autosampler, it is recommended to cool the samples at 10 °C until analysis; samples prepared in accordance with this standard have been checked to be stable at 10 °C for at least 24 h.

Column temperature control is essential (chromatography column oven or recirculating water jacket), if major drifts in retention times are to be avoided.

- f) UV detector set at 278 nm and 375 nm (optional). When using authentic TF standards, 375 nm is an option for some samples to get better chromatograms.

Ensure that the detector sensitivity range selected is able to keep all peaks from the highest mixed working standard F within the scale of the data collection system used.

### 9.6.3 HPLC analysis

Once the flow rate of the mobile phase (5.2) and temperature are stable, condition the column with a blank gradient run (9.6.2). Then inject onto the column 10 µl to 20 µl (for UHPLC: 1 µl to 10 µl) of each of the mixed standard solutions A to F (5.4) followed by an equal volume of the diluted test solution (9.5). Repeat the injection of the mixed working standard solutions at regular intervals (typically after six to 10 test solutions). Collect data using the data collection/integration system (6.12) for all peaks in the mixed standards and test sample solutions.

After each batch of analysis, thoroughly flush the chromatographic system and column with a volume fraction of 50 % acetonitrile (see 9.6.1) and replace column sealing plugs if disconnected for storage.

### 9.6.4 Identification

Identify the individual theaflavins by comparing retention times from sample chromatograms with those obtained from the mixed standard solutions obtained under the same chromatographic conditions (9.6.2). The use of diode array detection allows the UV profile of the theaflavin peaks to be scrutinised and peak purity assessed, which can be particularly useful for the determination of the low levels of theaflavins in black tea.

NOTE Where the availability of theaflavin marker compounds is limited, the analysis of a black leaf tea and a comparison with a typical HPLC chromatogram as well as UV-spectra and mass spectra given in Annex C will aid identification.

## 10 Calculation

### 10.1 General

Quantitation is performed by external standardization, using:

- either individual theaflavin standards of established purity and moisture content; or