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

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

1 Scope

This International Standard specifies a high performance liquid chromatographic (HPLC) or ultra-high performance liquid chromatographic (UHPLC) method for the determination of content of the four major theaflavins of tea, and is applicable to both leaf and instant black and oolong teas. The method is currently not validated for ready to drink 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 degrees C)*

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3 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 stabilise 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 9.1.2, Table 3).

4 Reagents

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

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

4.1 Water, grade 1 according to ISO 3696.

4.2 Acetonitrile, HPLC grade.

4.3 Methanol, HPLC grade.

4.4 Acetic acid, glacial HPLC grade.

4.5 Ascorbic acid, analytical grade

4.6 Ethylendiaminetetraacetic acid disodium salt, dihydrate (EDTA), analytical grade

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

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

4.8 20 %-acetonitrile stabiliser solution

Fill a 500 ml volumetric flask half with water (4.1). Add 10 ml of glacial acetic acid (4.4) and 100 ml of acetonitrile (4.2). Add 125 mg each of ascorbic acid (4.5) and EDTA (4.6) and fill up to the mark with water (4.1). This solution may be stored in the fridge for up to a week.

4.9 Leaf/Infusion stabilising solution

Weigh 0,0625 g EDTA (4.6) and 0,0625 g ascorbic acid (4.5) into a 25 ml volumetric flask and fill up to the mark with water (4.1) 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.

4.10 HPLC mobile phases

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

Transfer 40 ml of acetic acid (4.4) into a 2 l one-mark volumetric flask. Add sufficient water (4.1) to half fill the flask and mix well. Dilute to the mark with water, mix and filter (5.10).

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

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Transfer 20 ml of acetic acid (4.4) into a 1 l one-mark volumetric flask. Add approximately 400 ml acetonitrile (4.2), mix well and dilute to the mark with acetonitrile, mix again and filter.

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4.11 Stock standard solutions

4.11.1 General

If theaflavins[1)] 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 9.1.2, Table 3).

4.11.2 Preparation of individual theaflavin stock standard solutions

Weigh approximately 20 mg (exact weight recorded) of individual theaflavins (theaflavin: TF, theaflavin-3-gallate: TF-3-g, theaflavin-3'-gallate: TF-3'-g and theaflavin-3,3'-gallate: TF-3,3'-dig)) into separate 10 ml volumetric flasks. Fill up to mark with 20 % acetonitrile stabilising solution (4.6) 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.

1) Statistical data in the annex have been generated using the calibration against caffeine (see 4.11)

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4.12 Mixed standard solutions

To prepare the mixed standards pipette the following aliquots of the individual standards into 20 ml volumetric flasks and fill up to the mark with 20 % acetonitrile stabilising solution (4.6).

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 TF's given in Table 1 is based on 100 % purity of the standards. The purity of the standard TF's should be determined by high resolution NMR spectroscopy and the nominal concentrations of the standards accordingly to give the exact concentration of the individual TFs in each standard.

NOTE 1 With theaflavins of unknown purity it is essential that an individual HPLC assessment is first carried out to check for other potentially interfering components.

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NOTE 2 The nominal concentrations of the mixed standard solutions A-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.

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NOTE 3 The mixed working standard solutions A-F 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.

4.13 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 stabilising solution (4.6).

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 µg/10 µl
A	1,0 µg std	1	100	1,0
B	0,5 µg std	0,5	50	0,5
C	0,25 µg std	0,25	25	0,25
D	0,1 µg std	0,1	10	0,1
E	0,05 µg std	0,05	5	0,05

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Table 2 *(continued)*

Standard name	Individual stock caffeine standards diluted to 20 ml	Nominal concentration of caffeine	Nominal concentration of injection volume
	ml	mg/l	µg/10 µl
F 0,025 µg 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 non-linear calibration plot.

5 Apparatus

Usual laboratory apparatus and in particular the following.

5.1 Analytical balances, accuracy of ± 0,000 1 g and ± 0,000 01 g (see 4.9.2).

5.2 Water bath, of (70 ± 1) °C.

5.3 Dispenser, for methanol/water extraction mixture (4.5), and set at 5,0 ml.

5.4 Centrifuge, capable of 2000 Relative Centrifugal Force (typically 3 500 r.p.m.).

Micro centrifuge (14500 r.p.m) as an alternative to filtration (see 8.3).

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5.5 Vortex mixer, for efficient mixing during extraction

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5.6 Extraction tubes, glass, 10 ml capacity, stoppered and able to withstand centrifugation.

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

5.8 Volumetric flasks, 5 ml, 10 ml, 20 ml, 100 ml, 500 ml, 1 l and 2 l one-mark flasks.

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 and 0,2 µm (for UHPLC) pore size for filtration of mobile phases and diluted sample extracts.

NOTE PTFE and Nylon membrane filters have proven to be suitable, but all membranes have to be checked to ensure that theaflavin retention does not occur.

5.11 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 °C.

5.12 Data collection/integration system

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5.13 Chromatographic column for HPLC. Suitable RP columns for HPLC, dimensions e.g. 100 x 4.6. 150 x 4.6 or 250 x 4.6; i.d. of 2 mm is also possible (the flow has to be adjusted when using 2 mm columns, particle size 5, 3.5 or 3 µ. For UPLC typical dimensions are 100 x 2.1 mm, particle diameter 1.8 µ

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

6 Sampling

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

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

— ISO 1839 for leaf tea;

— 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.

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Grinding of instant tea is only required on samples of a coarse granular structure (for example freeze-dried instant tea).

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8 Procedure

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8.1 General

If it is required to check whether the repeatability requirement (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;

— ISO 7513 for instant tea.

8.3 Test portion

8.3.1 Instant tea

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

8.3.2 Leaf tea

Weigh (0,2 ± 0,001) g of the test sample (clause 7) into an extraction tube (5.6).

2) This is

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8.3.3 Brews

Pipette 4 ml tea infusion into a 5 ml volumetric flask (5.8), add 0,5 ml of acetonitrile (4.2) and fill up to the mark with leaf stabilising solution (4.7). This results in a dilution of 1,25.

Decant into extraction tubes (5.6) and centrifuge in a micro centrifuge at 14,500 rpm for 10 min and pipette supernatant into HPLC vials for analysis. Alternatively filter through a 0,45 µm or 0,2 µm filter (5.10).

NOTE Tea brews have not been validated in the ring test.

8.4 Extraction of theaflavins

8.4.1 Instant tea

Add to the instant tea in the flask (8.3.1) 20 % acetonitrile stabilising solution (4.6). Ensure that the sample has dissolved and sonicate if necessary. Fill up to the mark with 20 % acetonitrile stabilising solution (4.6), mix and filter (5.10), in case of UHPLC use a 0,2 µm filter.

8.4.2 Leaf tea

8.4.2.1 Place the methanol/water extraction mixture (4.5) contained in the dispenser (5.3) into the water bath (5.2) set at 70 °C, and allow at least 30 min for the extraction mixture to equilibrate.

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8.4.2.2 Place the extraction tube containing the sample (8.3.2) into 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).

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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.

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NOTE 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 to cooling to room temperature. Remove the stopper and place the tube in the centrifuge (5.4) at 3 500 r.p.m. 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, fill up to 10 ml with cold methanol/water extraction mixture (4.5) and mix the contents.

NOTE 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

If necessary, use a pipette and transfer 1,0 ml of the sample extract (instant tea extract from 8.4.1 or leaf tea extract from 8.4.2.6) into a graduated tube (5.7) and dilute to 2,0 to 5,0 ml with stabilising solution (4.7). In case of leaf tea extracts a degradation might occur on prolonged storage time prior to analysis if no stabilisation solution is used. Mix and filter (5.10), in case of UHPLC use 0,2 µm filter.

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8.6 Determination

8.6.1 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.

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:

1) Flow rate of the mobile phase (4.8): 2,0 ml/min; (for UHPLC 0.5-1 mL/min) both depending on the column used

2) conditions: 78 % mobile phase A (4.8.1) and 22 % mobile phase B (4.8.2); depending on the column used it might be necessary to adjust solvent composition slightly (e.g. 77/23 or 79/21)

3) run time: 25 min (UHPLC 8 - 10 min), both depending on the column used and the solvent composition;

4) temperature of the column (5.13): 30 °C ± 0,5 °C.

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5) When using a thermostatted autosampler it is recommended to cool the samples at 10 °C until analysis. Samples prepared according to this standard have been checked to be stable at 10 °C for at least 24 h.

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

6) 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.

8.6.3 HPLC analysis

Once the flow rate of the mobile phase (4.8) and temperature are stable, condition the column with a blank gradient run (8.6.2). Then inject onto the column 10 µl to 20 µl (UHPLC 1-10 µL) of each of the mixed standard solutions A-F (4.10) followed by an equal volume of the diluted test solution (8.5). Repeat 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 (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 a volume fraction of 50 % acetonitrile (see 8.6.1) and replace column sealing plugs if disconnected for storage.

8.6.4 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 (8.6.2). The use of diode array detection allows the UV profile of the theaflavin peaks to be

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