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TEA — DETERMINATION OF THEAFLAVINS IN BLACK TEA METHOD USING HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

SRI LANKA STANDARDS INSTITUTION

Sri Lanka Standard

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This Standard does not purport to include all the necessary provisions of a contract

Sri Lanka Standard
TEA — DETERMINATION OF THEAFLAVINS IN BLACK TEA METHOD USING HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

NATIONAL FOREWORD

This Sri Lanka Standard was approved by the Sectoral Committee on Food Products and was authorized for adoption and publication as a Sri Lanka Standard by the Council of the Sri Lanka Standards Institution on 2022-07-07

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.

This Sri Lanka Standard is identical with **ISO 18447 : 2021** Tea — Determination of theaflavins in black tea method using high performance liquid chromatography published by the International Organization for Standardization (ISO).

Terminology and conventions:

The text of the International Standard has been accepted as suitable for publication, without deviation, as a Sri Lanka Standard. However, certain terminology and conventions are not identical with those used in Sri Lanka Standards. Attention is therefore drawn to the following:

a) Wherever the words “International Standard” appear referring to this Standard, it should be interpreted as “Sri Lanka Standard”.

b) The comma has been used throughout as a decimal marker. In Sri Lanka Standards it is the current practice to use the full point on the base line as the decimal marker.

c) Wherever page numbers are quoted, they are ISO page numbers.

CROSS REFERENCES

International Standard	Corresponding Sri Lanka Standard
ISO 1572, Tea — Preparation of ground sample of known dry matter content	SLS 28-1 Preparation of ground sample of known dry matter content
ISO 3696, Water for analytical laboratory use — Specification and test methods	No corresponding Sri Lanka Standard
ISO 7513, Instant tea in solid form — Determination of moisture content (loss in mass at 103 °C)	SLS 1447 -2 Method of test for instant tea Determination of moisture content (loss in mass at 103 °C)

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INTERNATIONAL STANDARD

ISO 18447

First edition
2021-06

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

Thé — Détermination des théaflavines dans le thé noir — Méthode par chromatographie 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.

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).

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. Details of any patent rights identified during the development of the document will be in the Introduction and/or on the ISO list of patent declarations received (see www.iso.org/patents).

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.

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.

INTERNATIONAL STANDARD ISO 18447:2021(E)

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.

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 fill half of 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 the 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 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
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 nonlinear calibration plot.

6 Apparatus

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

6.1 Analytical balances, accuracy of ± 0,000 1 g and ± 0,000 01 g (See NOTE under 5.3.2).

6.2 Water bath, of (70 ± 1) °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 µm and 0,2 µm (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 °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 UHPLC, 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[1)] . For UHPLC, use, for example, an Eclipse Plus C18 RRHD, 1,8 µ, 100 mm × 2,1 mm[2)].

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).

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.

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.

2) This is an example of a suitable product 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 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.

9.3.2 Leaf tea

Weigh (0,2 ± 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 µm 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 µm 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 6 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

— a caffeine standard used in conjunction with consensus individual theaflavin RRFs measured with respect to caffeine (see 5.5. and 10.3).

NOTE Caffeine elutes very early in the chromatograms.

10.2 Quantitation using theaflavin standards

10.2.1 Calculate to the nearest 0,1 g/ml the concentration of anhydrous standard in each of the mixed standard solutions A to F (5.4).

10.2.2 Construct linear calibration graphs for each standard from the anhydrous concentrations (g/ml) against the peak areas obtained from the data collection/integration system (6.12) and obtain slope and intercept values.

10.2.3 The individual component content, expressed as a percentage by mass on a sample dry matter basis, is given by Formula (1):

$$\frac{\left(A_{\text{sample}} - A_{\text{intercept}}\right) \times V \times d \times 100}{S_{\text{Std}} \times m \times 10\ 000 \times D} \tag{1}$$

where

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

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

S_{Std} is the standard calibration line slope;

V is the sample extraction volume (in ml), typically for instant tea and for leaf tea: 10 ml;

d is the dilution factor (see 9.5), typically 1 to 5;

m is the mass of the sample test portion, in g;

D is the dry matter content, expressed as a mass fraction in per cent, of the test sample, determined in accordance with 9.2.

10.2.4 Total theaflavin content, *T*, as a percentage by mass on a sample dry matter basis, is given by the summation of the individual theaflavins.

$$T = T_1 + T_2 + T_3 + T_4$$

where

T_1 is the percentage of theaflavin content;

T_2 is the percentage of theaflavin-3-gallate content;

T_3 is the percentage of theaflavin-3′-gallate content;

T_4 is the percentage of theaflavin-3,3′-gallate content.

10.3 Quantitation using a caffeine standard and theaflavin RRFs

10.3.1 The RRF values (measured with respect to caffeine) of the individual theaflavins obtained from the international interlaboratory test are given in Table 3. 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. Information on the standards is provided in Annex B."

Table 3 — Consensus relative response factors at 278 nm

Component	RRF with respect to caffeine (calculated on standard *DM* basis) including the NMR purity factor
Theaflavin	0,779 3
Theaflavin-3-gallate	0,777 7
Theaflavin-3′-gallate	0,818 6
Theaflavin-3,3′-gallate	0,855 3

10.3.2 Construct a linear caffeine calibration graph from the anhydrous concentrations (in g/ml) against the caffeine peak areas obtained for each of the standard solutions A to F (5.4) and obtain the slope and intercept value.

10.3.3 The identified individual component (see 9.6.4) content, expressed as a percentage by mass on a sample dry matter basis, is given by Formula (2):

$$\frac{\left(A_{\text{sample}} - A_{\text{intercept}}\right) \times d \; x \; 100}{S_{\text{Caffeine}} \times m \times R_{\text{Std}} \times D \times 10\ 000} \tag{2}$$

where

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

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

S_{Caffeine} is the caffeine calibration line slope;

R_{Std} is the RRF, measured with respect to caffeine for the individual component;

V is the sample extraction volume in ml, typically for instant tea and for leaf tea: 10 ml;

d is the dilution factor (see 9.5), typically 1 to 5;

m is the mass, in grams of the sample test portion;

D is the dry matter content, expressed as a mass fraction in per cent, of the test sample, determined in accordance with 9.2.

For samples with very small peak areas and in cases where the intercept is high, it is better to use a calibration plot forced through origin, or even a one-point calibration plot with the standard of a comparable area.

10.3.4 Total theaflavin content, as a percentage by mass on a sample dry matter basis (RTD beverages in mg × l^{-1}), is given by the summation of the individual theaflavins.

$$T = T_1 + T_2 + T_3 + T_4$$

where

T_1 is the percentage of theaflavin content;

T_2 is the percentage of theaflavin-3-gallate content;

T_3 is the percentage of theaflavin-3′-gallate content;

T_4 is the percentage of theaflavin-3,3′-gallate content.

11 Precision

11.1 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.

11.2 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.

11.3 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.

12 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 document, i.e. ISO 18447:2021;

— all operating details not specified in this document, 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;

— the date of the test.

Annex A
(informative)

Results of interlaboratory tests

An interlaboratory test, carried out in 2019 under the auspices of ISO, gave the statistical results (evaluated in accordance with ISO 5725-2) shown in Table A.1.

The results in Table A.1 were obtained from quantitation using a caffeine standard in conjunction with consensus individual theaflavin RRFs. Total theaflavin content is expressed as a percentage, on an anhydrous standard and sample dry matter basis.

Table A.1 — Precision data

Sample identification	Sample 1: Black leaf tea	Sample 2: Black leaf tea	Sample 3: Black tea powder	Sample 4: Black tea powder
Number of participating laboratories	13	13	13	13
Number of accepted test results	11	12	9	10
Mean total theaflavin content, in % (mass fraction), dry matter	0,842	0,381	0,574	0,269
Repeatability standard deviation, s_r	0,019	0,008	0,016	0,010
Repeatability coefficient of variation, $C_{V,r}$, in %	2,2	2,2	2,7	3,7
Repeatability limit, r (2,8 × s_r)	0,053	0,024	0,044	0,028
Reproducibility standard deviation, s_R	0,191	0,075	0,166	0,074
Reproducibility coefficient of variation, $C_{V,R}$, in %	22,7	19,7	28,9	27,5
Reproducibility limit, R (2,8 × s_R)	0,535	0,211	0,465	0,207

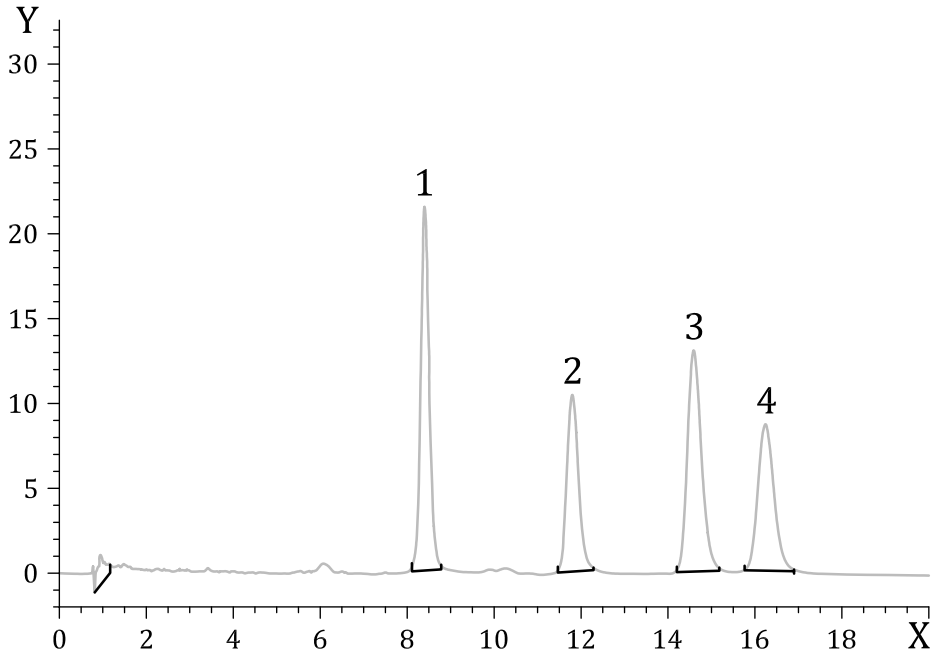
Annex B
(informative)

Assessment of the purity of standards used in the RRF work

Component	HPLC area %	UHPLC area %	NMR %
Theaflavin	96,7	96,4	87
Theaflavin-3-gallate	92,5	91,5	80
Theaflavin-3-gallate	91,4	90,7	83
Theaflavin-3,3′-gallate	93,3	92,0	82

Annex C
(informative)

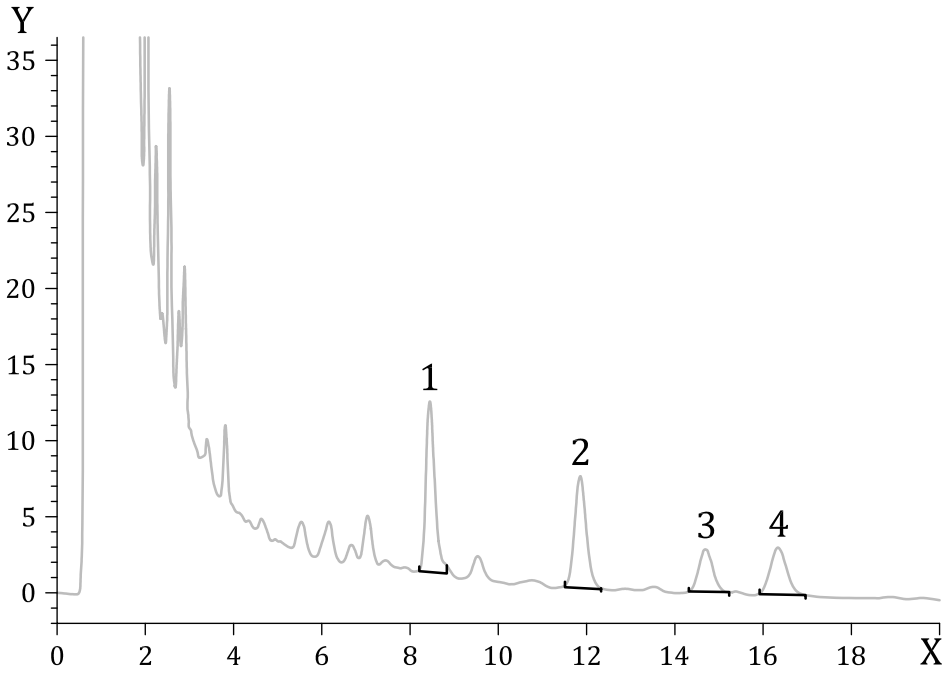
Typical HPLC chromatograms



Key

X time *t*, min
Y intensity, mAU
1 theaflavin (TF)
2 theaflavin-3-gallate (TF-3-g)
3 theaflavin-3′-gallate (TF-3-gallate (TF-3′-g)
4 theaflavin-3,3′ gallate (TF-3,3′-g)

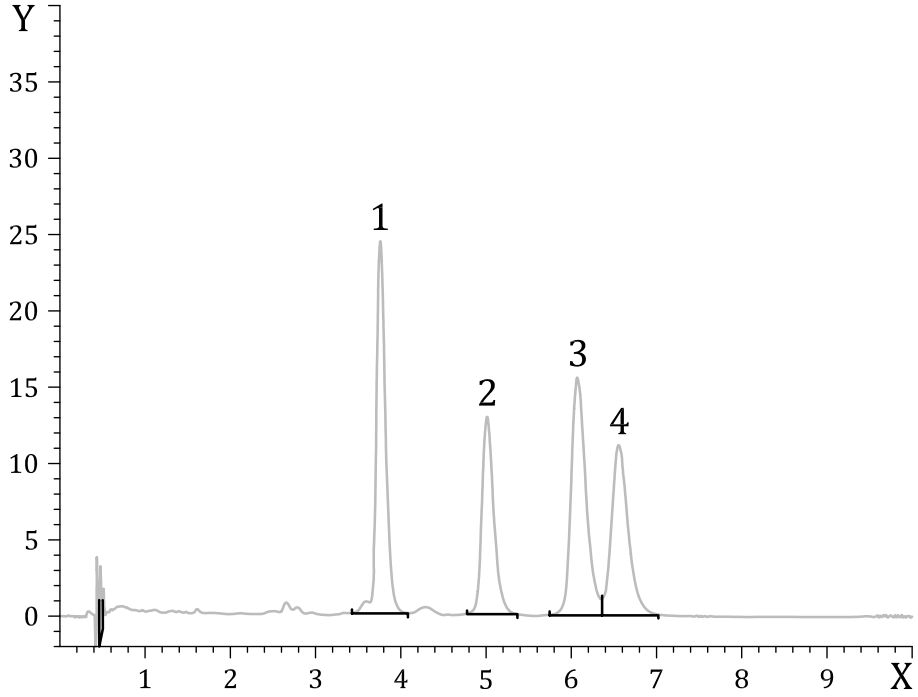
Figure C.1 — Mixed theaflavin standard on HPLC



Key

X time *t*, min
Y intensity, mAU
1 theaflavin (TF)
2 theaflavin-3-gallate (TF-3-g)
3 theaflavin-3′-gallate (TF-3-gallate (TF-3′-g)
4 theaflavin-3,3′ gallate (TF-3,3′-g)

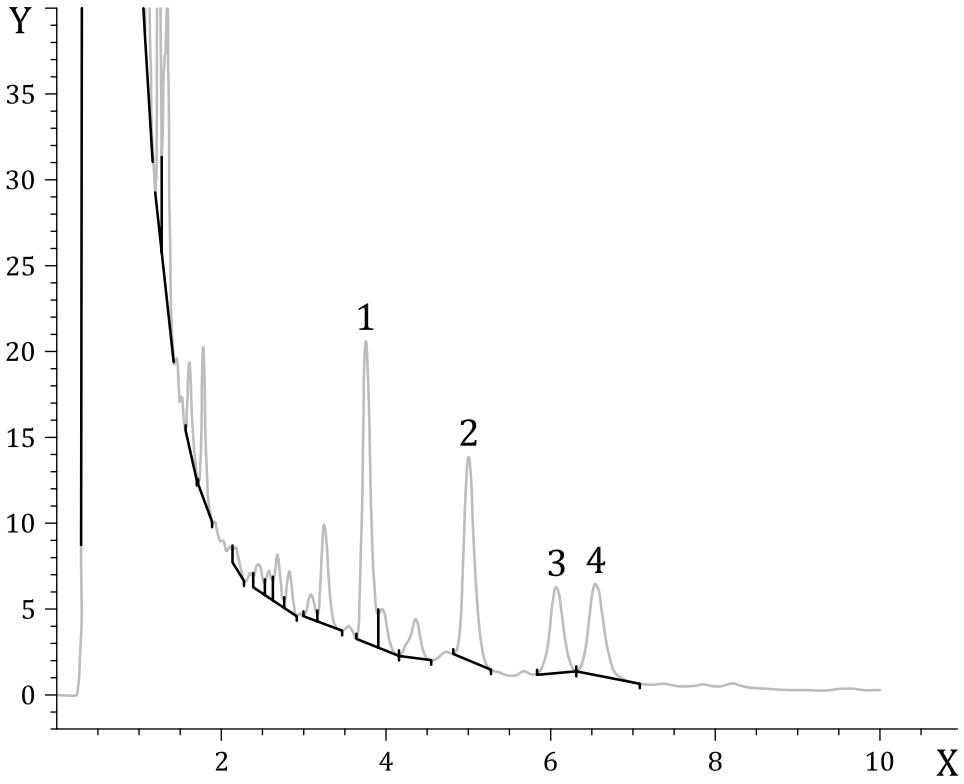
Figure C.2 — Black tea powder on HPLC



Key

X time *t*, min
Y intensity, mAU
1 theaflavin (TF)
2 theaflavin-3-gallate (TF-3-g)
3 theaflavin-3′-gallate (TF-3-gallate (TF-3′-g)
4 theaflavin-3,3′ gallate (TF-3,3′-g)

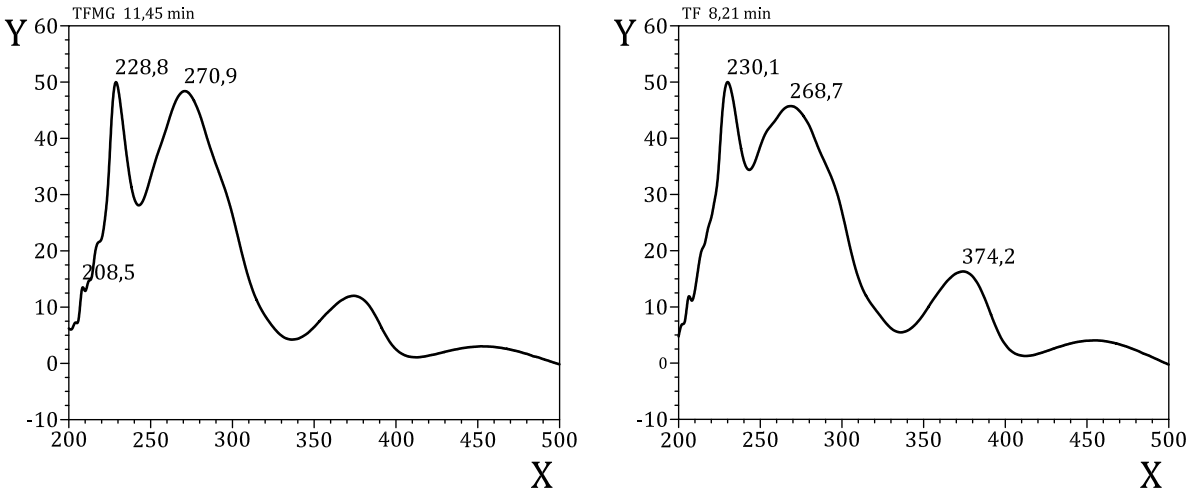
Figure C.3 — Mixed theaflavin standard on UHPLC



Key

X time *t*, min
Y intensity, mAU
1 theaflavin (TF)
2 theaflavin-3-gallate (TF-3-g)
3 theaflavin-3′-gallate (TF-3-gallate (TF-3′-g)
4 theaflavin-3,3′ gallate (TF-3,3′-g)

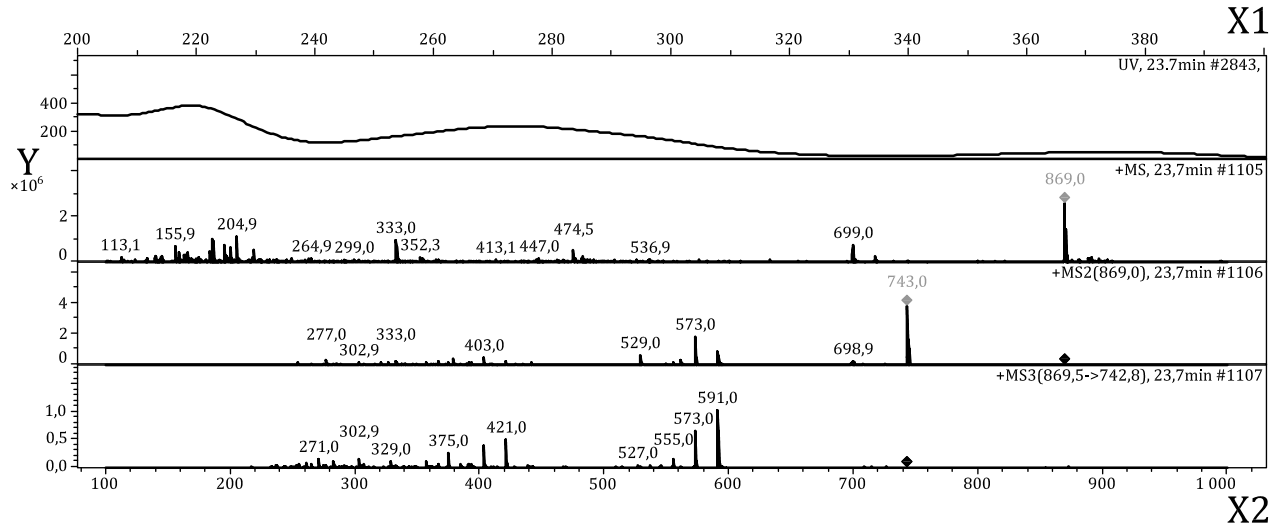
Figure C.4 — Black tea powder on UHPLC



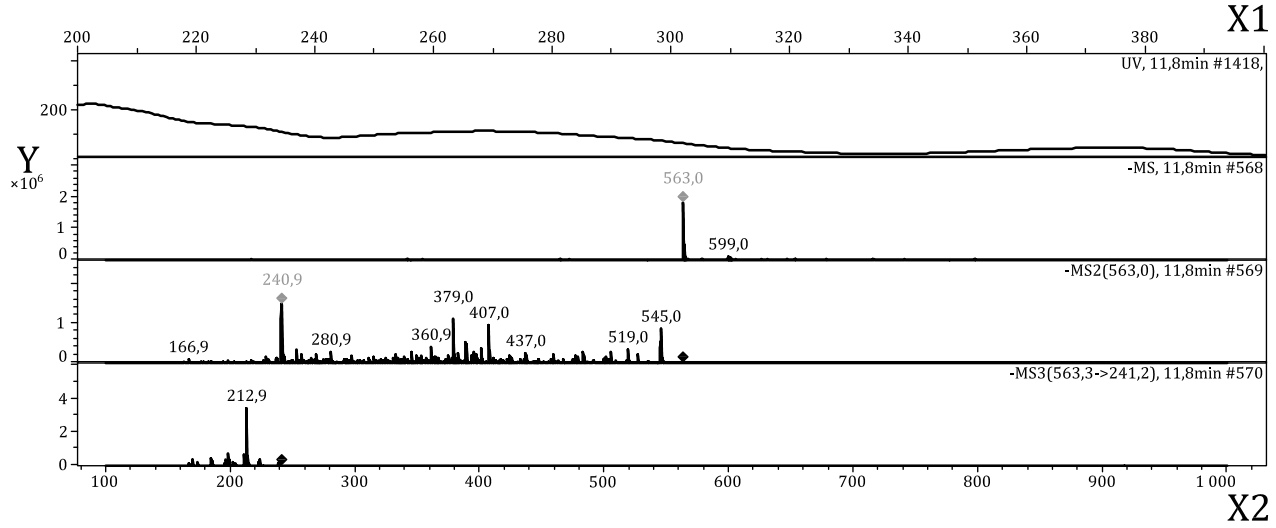
Key

X wavelength, nm
Y relative intensity, *r*

Figure C.5 — UV-Spectra of theaflavins



a) Theaflavin-3-3′-digallate



b) Theaflavin

Key

X1 wavelength, nm

X2 m/z

Y intensity, mAU

Figure C.6 — Mass spectra of theaflavins

Bibliography

[1] ISO 1839, *Tea — Sampling*

[2] ISO 5725-2, *Accuracy (trueness and precision) of measurement methods and results — Part 2: Basic method for the determination of repeatability and reproducibility of a standard measurement method*

[3] ISO 7516, *Instant tea in solid form — Sampling*

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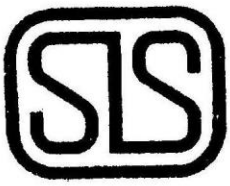

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