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Coffee and coffee products — Determination of the caffeine content using high performance liquid chromatography (HPLC) — Reference method

Café et dérivés du café — Détermination de la teneur en caféine par chromatographie liquide à haute performance (CLHP) — Méthode de référence

Reference number
ISO 20481:2008(E)

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Foreword

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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 20481 was prepared by Technical Committee ISO/TC 34, *Food products*, Subcommittee SC 15, *Coffee*.

This corrected version of ISO 20481:2008 incorporates the following corrections:

a) from Clause 2, ISO 565, ISO 648, and ISO 1042 have been moved to the bibliography, and the numbering of the bibliographic references and their citations adjusted accordingly throughout;

b) in 8.3, paragraph 1, the second mention of “8.2.1” has been deleted, and “8.2.2” inserted;

c) in 9.1, “w_x” (2 occurrences) has been deleted, and “w_{c}” inserted;

d) in 9.1 and 9.2, “A_{c}” (5 occurrences) has been deleted, and “A_{st}” inserted;

e) in 9.1 and 9.2, “A_x” (5 occurrences) has been deleted, and “A_{s}” inserted;

f) in 9.1 and 9.2, “ρ_{c}” (5 occurrences) has been deleted, and “ρ_{st}” inserted;

g) in 9.2, “ w'_x ” (2 occurrences) has been deleted, and “ w'_{c} ” inserted;

h) in 9.2, a comma has been inserted after w'_{c} ;

i) in Table A.1, row 7 (Standard deviation of repeatability, s_r), column 13 (Soluble coffee, Regular agglomerated), “0,30” has been deleted, and “0,030” inserted.

Coffee and coffee products — Determination of the caffeine content using high performance liquid chromatography (HPLC) — Reference method

1 Scope

This International Standard specifies a high performance liquid chromatography (HPLC) method for the determination of the caffeine content of: green coffee; roasted coffee; soluble coffee, regular and decaffeinated; and mixed instant coffee products (e. g. coffee/chicory mix or cappuccino-type coffee drink).

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 3696, *Water for analytical laboratory use — Specification and test methods*

ISO 3726, *Instant coffee — Determination of loss in mass at 70 °C under reduced pressure*

ISO 6673, *Green coffee — Determination of loss in mass at 105 °C*

ISO 11817, *Roasted ground coffee — Determination of moisture content — Karl Fischer method (Reference method)*

3 Principle

Caffeine is extracted from samples with water at 90 °C in the presence of magnesium oxide. After filtration, the caffeine content of the extract is determined by HPLC on a RP-18 column using isocratic elution with UV detection at approximately 272 nm.

Wherever appropriate, the caffeine content may be given on dry basis which requires a moisture determination by a suitable standard method.

4 Reagents

Unless otherwise specified, use only reagents of recognized analytical grade, and only water conforming to the requirements of ISO 3696, grade 1.

4.1 Methanol, HPLC grade.

4.2 Magnesium oxide (MgO), heavy, high grade [1].

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[1] Merck 105867 is an example of a suitable, commercially available product. This information is given for the convenience of users of this International Standard and does not constitute an endorsement of this product by ISO.

NOTE The use of MgO is important for the lifetime of the analytical column, especially for green coffee. The amount of MgO used depends on the equipment and on the type of coffee product. The precision data in Annex A were acquired using the procedure described in this International Standard.

The appearance of interfering peaks in the chromatogram may be due to improper adsorption. In these cases, subject the MgO used to examination.

4.3 Caffeine (1,3,7-trimethylxanthine; 1,3,7-trimethyl-1*H*-purine-2,6(3*H*,7*H*)-dione; methyltheobromine; $C_8H_{10}N_4O_2$), pure anhydrous.

4.4 Mobile phase, 24 % volume fraction methanol in water.

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

Transfer 240 ml methanol (4.1) to a 1 l one-mark volumetric flask (5.11). Make up to the mark with water, mix and filter through a 0,45 µm filter (5.2).

NOTE By changing the ratio of methanol to water, the retention time of caffeine can be adjusted to optimize the HPLC separation on the column used.

4.5 Caffeine standard solutions.

4.5.1 Stock solution, corresponding to 200 mg/l.

Weigh (0,200 ± 0,001) g anhydrous caffeine (4.3) into a 1 l one-mark volumetric flask (5.11). Add sufficient warm water to half fill the flask. Swirl to dissolve the caffeine, cool to room temperature, make up to the mark with water and mix.

The solution is stable at +4 °C for one month. Store in a refrigerator.

4.5.2 Dilute standard solution for regular coffee, corresponding to approximately 40 mg/l.

Pipette (5.12) 50 ml of the caffeine standard stock solution (4.5.1) to a 250 ml one-mark volumetric flask (5.11). Make up to the mark with water and mix. Prepare fresh dilute standard solution daily.

4.5.3 Dilute standard solution for decaffeinated coffee, corresponding to approximately 4 mg/l.

Pipette (5.12) 5 ml of the caffeine standard stock solution (4.5.1) to a 250 ml one-mark volumetric flask (5.11). Make up to the mark with water and mix. Prepare fresh dilute standard solution daily.

4.5.4 Calibration plot. The use of a three to five point calibration plot is optional. Recommended concentration range is 5 mg/l to 25 mg/l for regular coffee and 0,5 mg/l to 2,5 mg/l for decaffeinated samples.

5 Apparatus

Usual laboratory apparatus and, in particular, the following.

5.1 Analytical balance, capable of weighing to an accuracy of ± 0,1 mg.

5.2 Membrane filter units of pore size 0,45 µm, for filtration of mobile phases and diluted sample extracts.

5.3 High performance liquid chromatograph, equipped to perform an isocratic elution, with a UV detector set at 272 nm (270 nm to 280 nm) or a filter detector (254 nm), and with a data collection/integration system. The use of a degasser is optional.

5.4 Chromatographic column for HPLC, of minimum length 125 mm, packed with C18 material, preferentially 5 µm spherical particles, with an efficiency of separation of at least 5 000 theoretical plates. The theoretical number of plates, N_{th}, of the column may be calculated by determining peak shape after injection of the caffeine standard solution by Equation (1):

$$N_{\text{th}} = \left(\frac{t_{\text{r}}}{b}\right)^2 \times 5{,}54 \tag{1}$$

where

t_{r} is the retention time, in seconds, of the peak;

b is the peak width, in seconds, at half peak height.

5.5 Magnetic stirrer, with heater and mounted water bath.

5.6 Ultrasonic bath.

5.7 Microlitre syringe.

5.8 Coffee mill, suitable for grinding roasted coffee beans.

5.9 Grinder with cogged wheel, with cooling jacket, or **analytic grinder**, with blades and cooling jacket, or any other device suitable for grinding green coffee beans.

5.10 Sieve, of nominal size of openings 630 µm, complying with the requirements of ISO 565[2], R20 series.

5.11 One-mark volumetric flasks, of capacities 1 l and 250 ml, complying with the requirements of ISO 1042[4], class A.

5.12 Pipettes, of capacities 50 ml and 5 ml, complying with the requirements of ISO 648[3], class A.

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 International Standard. The sampling procedure shall be subject to agreement by the interested parties.

Store the test sample in such a way that deterioration and change in its composition are prevented.

In case of mixed beverage powders, a minimum of 50 g of the product is required (for portion packages: a minimum of five portions).

7 Preparation of test samples

7.1 Green coffee

Grind (5.9) green coffee beans so that more than 50 % mass fraction of the sample passes through the sieve (5.10). Then take the test sample from the well-mixed total ground coffee.

7.2 Roasted coffee

Mill (5.8) roasted coffee beans so that more than 50 % mass fraction passes through the sieve (5.10).

Use roasted ground coffee in commercial packages without further treatment except homogenization.

7.3 Soluble coffee

Grinding of soluble coffee is not required.

7.4 Mixed beverages

Homogenize mixed coffee beverage powders using the mill (5.8) immediately before extraction in order to prevent segregation.

8 Procedure

8.1 Optional determination of coffee dry matter (see 9.2)

Calculate the coffee dry matter content to be used in 9.2 from the moisture content determined on a portion of the test sample (Clause 7) in accordance with

- ISO 6673 for green coffee,
- ISO 11817 for roasted coffee,
- ISO 3726 for instant coffee.

For other types of coffee and coffee-derived products, the dry matter content determination shall be subject to agreement between the interested parties.

8.2 Test portion

8.2.1 Roasted and green coffee

Weigh (5.1) about 1 g of the test sample (7.1, 7.2) to the nearest milligram, and transfer into a 250 ml volumetric flask (5.11).

8.2.2 Instant coffee

Weigh (5.1) about 0,5 g of the test sample (7.3) to the nearest milligram, and transfer into a 250 ml volumetric flask (5.11).

8.2.3 Mixed beverages

Weigh (5.1) about 1 g of the test sample (7.4) to the nearest milligram, and transfer into a 250 ml volumetric flask (5.11).

8.3 Extraction of caffeine

Add to the test portion (8.2.1, 8.2.2 or 8.2.3) in the flask, 5 g magnesium oxide (4.2) and about 200 ml water. Place the flask in a water bath maintained at boiling point and wait until the solution has reached at least 90 °C. Continue heating the flask in the water bath for 20 min, mix occasionally by shaking or stirring. Remove the volumetric flask from the water bath, cool to room temperature under tap water, and make up to volume with water. Wait until the solids have settled.

Take an aliquot of the supernatant solution, and filter through a 0,45 µm filter (5.2), discarding the first few millilitres. The filtrate is now ready for the HPLC separation.

8.4 Determination

8.4.1 Adjustment of the apparatus

IMPORTANT — The mobile phase should be degassed for 20 min in the ultrasonic bath (5.6) or purged with helium prior to use. If the HPLC equipment includes a degasser, 10 min are sufficient.

Set up the chromatograph (5.3) in accordance with the manufacturer's instructions and adjust it as follows:

a) flow rate of the mobile phase (4.4): 1,0 ml/min;

b) UV detector set at 272 nm (or 254 nm in case of a filter detector). Ensure that the detector sensitivity range fits to the peak of the standard solution (in case of more than one standard to the peak of the highest one).

8.4.2 HPLC analysis

Once the flow rate of the mobile phase and the corresponding pressure are stable, allow the system to equilibrate for at least 10 min. Then inject 10 µl of the standard solution onto the column, using the microlitre syringe (5.7), in the next run followed by an equal volume of the sample extract (8.3). When a single standard solution is used, inject it at regular intervals (typically after six sample extracts). After each batch of analysis, thoroughly flush the chromatographic system and column with 50 % volume fraction methanol (4.1) and water, and replace column sealing plugs if disconnected for storage.

9 Calculation

9.1 Caffeine content in the sample

Calculate the caffeine content, w_c, expressed as a percentage by mass, numerically equivalent to grams per 100 g coffee as is, according to Equation (2):

$$w_\mathrm{c} = \frac{A_\mathrm{s}\rho_\mathrm{st}V \times 100}{A_\mathrm{st}m_\mathrm{s}} = \frac{A_\mathrm{s}\rho_\mathrm{st} \times 25}{A_\mathrm{st}m_\mathrm{s}} \qquad (2)$$

where

A_st is the area, in arbitrary area units, of the HPLC caffeine peak of the caffeine standard solution;

A_s is the area, in arbitrary area units, of the HPLC caffeine peak of the sample solution;

m_s is the mass, in grams, of the test portion;

V is the volume, in litres, of the extracted sample solution, as defined in 8.2 (V = 0,25 l);

ρ_st is the mass concentration, in grams per litre, of the caffeine standard solution.

NOTE Peak height can be used as an alternative to peak area.

If more than one calibration standard is used, construct a linear calibration graph for the caffeine standards, to obtain the slope and intercept value. Use this calibration plot to calculate the concentration of caffeine in the sample.

Take as the result the arithmetic mean of two determinations carried out under repeatability conditions (10.2).

9.2 Optional determination of caffeine content in the sample on the dry basis (see 8.1)

Calculate the caffeine content, w'_c, expressed as a percentage by mass, numerically equivalent to grams per 100 g on the dry basis, by Equation (3):

$$w'_\mathrm{c} = \frac{A_\mathrm{s} \rho_\mathrm{st} V \times 100}{A_\mathrm{st} m_\mathrm{s} w_\mathrm{d}} \times 100 = \frac{A_\mathrm{s} \rho_\mathrm{st} \times 2\,500}{A_\mathrm{st} m_\mathrm{s} w_\mathrm{d}} \qquad (3)$$

where w_d is the dry matter content of the sample (grams per 100 g), as determined in accordance with 8.1.

Take as the result the arithmetic mean of two determinations carried out under repeatability conditions (10.2).

10 Precision

10.1 Interlaboratory test

Details of the interlaboratory test to determine the precision of the method are summarized in Annex A. The caffeine results were taken based on coffee as is.

The repeatability and reproducibility limits, as defined in ISO 5725-1:1994[5], 3.16 and 3.20, respectively, are calculated according to ISO 5725-6:1994[7], 4.1.4.

For the estimate of the Horwitz reproducibility limit, the Horwitz function, as given in Reference [9], was used.

The values derived from this interlaboratory test may not be applicable to concentration ranges and matrices others than those given.

Precision data evaluated in accordance with ISO 5725-1[5] are given in Table 1.

Table 1 — Means, repeatability limits and reproducibility limits

Sample/product	**Mean** % mass fraction	**Repeatability limit** r % mass fraction	**Reproducibility limit** R % mass fraction
Green coffee			
Decaffeinated (decaf)	0,057	0,004	0,009
Decaf	0,075	0,003	0,006
Regular arabica	1,14	0,05	0,15
Regular robusta	2,18	0,096	0,37
Roasted coffee			
Decaf	0,055	0,007	0,026
Mixture regular/decaf	0,642	0,054	0,091
Regular	1,22	0,053	0,176
Coffee drink powder			
Cappuccino type	0,403	0,024	0,068
Soluble coffee			
Decaf	0,072	0,004	0,024
Decaf	0,109	0,008	0,030
Regular freeze dried	2,51	0,050	0,23
Regular agglomerated	3,19	0,085	0,372
Soluble mix			
Coffee/chicory	2,45	0,066	0,216

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, may not exceed the repeatability limits, r, given in Table 1 in more than 5 % of cases (i.e. repeatability at a 95 % probability).

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, may not exceed the reproducibility limits, R, given in Table 1 in more than 5 % of cases (i.e. reproducibility at a 95 % probability).

11 Test report

The test report shall specify at least the following information:

a) all information required for the complete identification of the sample;

b) the method used, with reference to this International Standard;

c) the test result obtained;

d) if the repeatability has been checked, the final quoted result obtained;

e) any operating details not specified in this International Standard, or regarded as optional, as well as any circumstances that may have influenced the result.

Annex A
(informative)

Precision data of validation study on caffeine determination in coffee and coffee products