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Determination of theanine in tea and instant tea in solid form using high-performance liquid chromatography

Détermination de la théanine dans le thé et le thé instantané sous forme solide en utilisant la chromatographie en phase liquide à haute performance

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ISO copyright office
Ch. de Blandonnet 8 • CP 401
CH-1214 Vernier, Geneva, Switzerland
Tel. +41 22 749 01 11
Fax +41 22 749 09 47
copyright@iso.org
www.iso.org

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Foreword

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The committee responsible for this document is ISO/TC 34, *Food products*, Subcommittee SC 8, *Tea*.

Introduction

Theanine (N-ethyl-γ-L-glutamine) is a non-protein derived (free) amino acid. In the plant world, it is a unique amino acid found in *Camellia* genus and the mushroom *Boletus badius* and *Ilex guayusa*. The most common and the most prominent occurrence is in *Camellia sinensis* (L.) O. Kuntze species.

Theanine constitutes *circa* 0,1 % to 2 % w/w of the dry weight in tea leaves (*Camellia sinensis*) and is approximately 50 % of the total free amino acids. Separation of L- and D-theanine by HPLC is not achieved by this method; however, L-theanine is the major form (more than 95 %) in all teas[2][3]. Theanine has been associated with the typical tea umami taste and is also being studied to evaluate potential beneficial effects to health.

The umami taste characteristic has been associated with theanine which is also being evaluated for its potential benefits to health. This is also creating interest in theanine as an important novel food and dietary supplement ingredient.

Determination of theanine in tea and instant tea in solid form using high-performance liquid chromatography

1 Scope

This document specifies a high-performance liquid chromatographic (HPLC) method for the determination of theanine content in tea (*Camellia sinensis*). It is applicable to both tea and instant tea samples. Separation of L- and D-theanine is not possible using this method; however, the L-enantiomer is the major form in tea.

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 1573, *Tea — Determination of loss in mass at 103 °C*

ISO 1839, *Tea — Sampling*

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)*

ISO 7516, *Instant tea in solid form — Sampling*

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:

— IEC Electropedia: available at http://www.electropedia.org/

— ISO Online browsing platform: available at http://www.iso.org/obp

4 Principle

A reversed-phase (RP)-C18 column is used which is capable of separating more polar compounds using water as an eluent and UV detection at 210 nm. Theanine is separated from the other amino acids; however, the potential co-elution of theanine with methionine is not relevant due to the very small concentration of methionine in tea. It is recommended that polyamide column extract clean-up is used to remove interfering polyphenols for column protection when separation of theanine from other constituents in the extract sample is difficult. Alternatively, the column should be washed after each tea sample using acetonitrile.

5 Reagents

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

5.1 Water, conforming to grade 1 of ISO 3696.

5.2 Acetonitrile, HPLC grade (however, super grade is preferable).

5.3 Methanol, regular laboratory distilled grade.

5.4 Polyamide (Polyamide CC 6[1)], Particle size: 0,05 mm to 0,16 mm for column chromatography).

5.5 L-theanine reference standard, >99 %, anhydrous.

5.6 HPLC mobile phases.

WARNING — Wear gloves and eye protection and dispense reagents in a fume cupboard.

5.6.1 Mobile phase A, 100 % purified water (5.1).

5.6.2 Mobile phase B, 100 % acetonitrile (5.2).

5.7 Standard solution.

5.7.1 Standard stock solution, corresponding to 1 mg/ml.

Weigh (0,050 0 ± 0,000 1) g of pure L-theanine (5.5) in a 50 ml volumetric flask dissolve with purified water using sonication to aid dissolution and make up to the volume with purified water.

5.7.2 Standard working solution.

The stock solution is diluted with purified water to prepare the standard solutions in the concentration range of 5 µg/ml to 200 µg/ml as detailed in Table 1.

Table 1 — Stock solution dilutions

Standard solution	**Working solution** (µg/ml)	**Volume taken from the stock solution** (ml)	**Final volume** (ml)
A	5	0,5	100
B	10	1,0	100
C	20	1,0	50
D	50	2,5	50
E	100	5,0	50
F	150	7,5	50
G	200	10,0	50

1) Polyamide CC 6 is the required/minimum specification required for the separation which is available commercially. This information is given for the convenience of users of this document and does not constitute an endorsement by ISO of this product.

6 Apparatus

Usual laboratory apparatus and, in particular, the following:

6.1 Analytical balances, capable of weighing to an accuracy of ±0,000 1 g.

6.2 Magnetic stirrer, capable of 500 r/min.

6.3 Filters, membrane filter units of 0,45 µm pore size for filtration of mobile phase and diluted sample extracts. All membranes used should be checked using a simple calibration solution to ensure that theanine retention does not occur.

6.4 Centrifuge, capable of 12 300 relative centrifugal force (R.C.F.) (typically 13 500 r/min).

6.5 Glass column, 17 cm × 2,2 cm in diameter, (for polyamide clean-up only).

6.6 One mark volumetric flasks, of capacities 50 ml and 100 ml.

6.7 High performance liquid chromatograph, equipped to perform binary gradient elution, with a thermostatically controlled column compartment and an ultraviolet detector set at 210 nm.

6.8 Chromatographic column for HPLC, Phenomenex Aqua™[2)] 250 × 4,6 in diameter or 2 mm in diameter or equivalent.

NOTE This method has been developed with the Aqua™ which has the minimum specification required for this analysis.

7 Sampling

The sampling methods that shall be used are detailed in ISO 1839 for tea or ISO 7516 for instant tea.

A representative sample(s) shall be sent to the laboratory. It shall not be damaged or changed during transport or storage.

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 samples is only required with samples having a coarse granular structure (e.g. freeze-dried instant tea).

9 Procedure

9.1 General

If it is required to check whether the results are within the acceptable repeatability limit (see 11.2), carry out two single determinations in accordance with 9.2 to 9.4 under repeatability conditions.

2) Phenomenex Aqua™ is the required/minimum specification required for the separation which is available commercially. This information is given for the convenience of users of this document and does not constitute an endorsement by ISO of this product.

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 (Clause 8) in accordance with ISO 1573 for tea or ISO 7513 for instant tea.

9.3 Test portion

9.3.1 Sample preparations

9.3.1.1 Leaf tea

9.3.1.1.1 Weigh (1,00 ± 0,01) g of a finely ground sample into a 200 ml beaker and add 100 ml of boiling water.

9.3.1.1.2 Allow brewing for 5 min using a magnetic stirrer and then filter through a filter paper (Grade 4 or equivalent) into a 100 ml volumetric flask.

9.3.1.1.3 Allow the tea brew to cool down to ambient temperature, make up to volume of 100 ml with purified water and filter using a 0,45 µm membrane before injection. Alternatively, approximately 1 ml of the sample solution can be centrifuged at 13 000 r/min for 10 min prior to HPLC analysis.

9.3.1.2 Tea extract or instant tea

9.3.1.2.1 Weigh (0,100 ± 0,001) g sample into a 50 ml volumetric flask, dissolve and make up with heated purified water (approximately 50 °C).

9.3.1.2.2 Sonicate for 2 min to 5 min for complete dissolution and filter using a 0,45 µm membrane before injection. Alternatively, approximately 1 ml of the sample solution can be centrifuged at 13 000 r/min for 10 min prior to HPLC analysis.

9.3.2 Sample clean-up (using a polyamide column) — Optional

Certain tea samples will not elute with clean chromatograms. When this occurs in the position where theanine elutes in the chromatogram, the interference will prevent an accurate determination of the peak area. On these occasions, it is necessary to carry out the clean-up procedures as described below in order to improve separation and to protect the column.

9.3.2.1 Suspend 1,2 kg polyamide in a mixture of 8 l of water and 1,5 l of methanol, mix well and allow settling overnight.

9.3.2.2 Remove supernatant and wash with 1,5 l methanol twice. Store in methanol and mix well before use.

9.3.2.3 Column chromatography:

9.3.2.3.1 Fill a glass column (17 cm × 2,2 cm in diameter) equipped with a thin layer of glass wool and sea sand with swollen polyamide to a height of 8 cm. Condition with 250 ml of water.

9.3.2.3.2 Apply 50 ml of the tea solution to the column and immediately collect the eluate into a 100 ml volumetric flask.

9.3.2.3.3 Wash the column with purified water until the mark of 100 ml is reached. Mix well and filter using a 0,45 µm membrane before injection. Alternatively, approximately 1 ml of the sample solution can be centrifuged at 13 000 r/min for 10 min prior to HPLC analysis.

The polyphenols will be retained on the polyamide while the theanine elutes. This step corresponds to a twofold dilution.

9.4 Determination

9.4.1 Adjustment of the apparatus

Set up the chromatograph (6.7) in accordance with the manufacturer's instructions and adjust it as follows.

a) Flow rate of the mobile phase (5.6.1 and 5.6.2): 1,0 ml/min for 4,6 mm columns and 0,25 ml/min for 2 mm columns.

b) Binary gradient conditions: according to the elution programme as detailed in Table 2.

c) Temperature of the column (6.8): ambient, optional 20 °C.

d) UV detector set at 210 nm.

e) Injection volume: 20 µl (optional 10 µl to 50 µl).

Table 2 — Elution programme

Time (min)	% **A**	% **B**	**Comment**
0	100	0	analysis
10	100	0	analysis
12	20	80	wash
20	20	80	wash
22	100	0	conditioning
40	100	0	conditioning

9.4.2 HPLC analysis

Once the flow rate of the mobile phase (5.6) and temperature [see 9.4.1 c)] are stable, condition the column with a blank gradient run (9.4.1). Then inject onto the column 20 µl of each of the standard working solutions (5.7.2), followed by an equal volume of the sample solution (9.3.1.1.3 or 9.3.1.2.2). Repeat the injection of the mixed working standard solutions (5.7.2) at regular intervals (typically after six test solutions). Collect data using the data collection/integration system of the HPLC (6.5) for the peak of theanine in the standard and test sample solutions.

After each batch of analysis, thoroughly clean the column by flushing the chromatographic system and column with 50 % acetonitrile, 50 % water mobile phase. Replace column sealing plugs if disconnected for storage.

9.4.3 Identification

Identify the theanine by comparing retention time from sample chromatograms with that obtained from the standard solutions under the same chromatographic conditions (see 9.4.1).

NOTE Typical chromatograms can be found in Annex B.

10 Calculation

Draw a theanine calibration graph by using concentration of theanine in the working solutions (5.7.2) against theanine peak area.

Obtain the calibration line slope and intercept value (m and b as in $y = mx + b$). A typical theanine calibration graph is given in Figure A.1.

A linear calibration should be obtained. The theanine content, W_{theanine}, expressed as a percentage by mass on a sample dry matter basis, is given by Formula (1):

$$W_{\text{theanine}} = [(A_{\text{sample}} - b_{\text{intercept}}) \times V_{\text{sample}} \times d \times 100]/[m_{\text{std}} \times M_{\text{sample}} \times 10\,000 \times w_{\text{DM, sample}}] \quad (1)$$

where

A_{sample} is the peak area obtained for the sample test solution;

$b_{\text{intercept}}$ is the y-intercept;

m_{std} is the slope obtained from the best-fit linear calibration;

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

d is the dilution factor used prior to the injection on to an HPLC;

$w_{\text{DM, sample}}$ is the dry matter content, expressed as a mass fraction in percent, of the test sample, determined in accordance with ISO 7513 or ISO 1573.

V_{sample} is the extraction volume (in ml).

11 Precision

11.1 Interlaboratory test

Details of the interlaboratory test to determine the precision of the method are summarized in Table C.1. 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 no more than 5 % of cases be greater than the repeatability limit values given in Table C.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 no more than 5 % of cases be greater than the reproducibility limit values given in Table C.1.

12 Test report

The test report shall specify the following:

— all information necessary for the complete identification of the sample and its source;

— the sampling method used: ISO 1839 for tea, ISO 7516 for instant tea, or another if applicable;

— the specific test method used, with reference to this document;

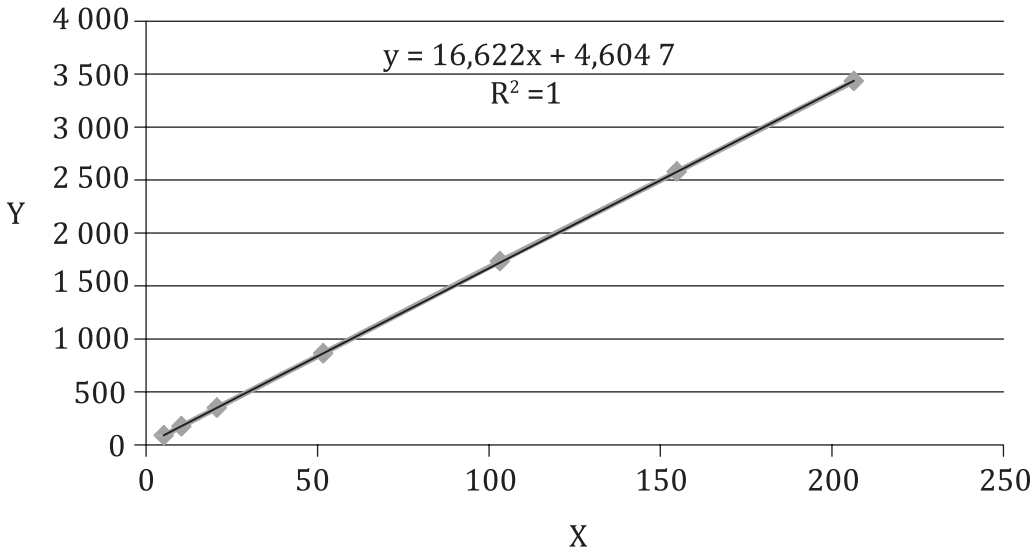
— all operating details not specified in this document, or regarded as optional, together with details of any incidents which may have influenced the test result(s);

— the test result(s) obtained;

— if the repeatability has been checked, the final quoted result obtained.

Annex A
(informative)

Typical theanine calibration graph



Key

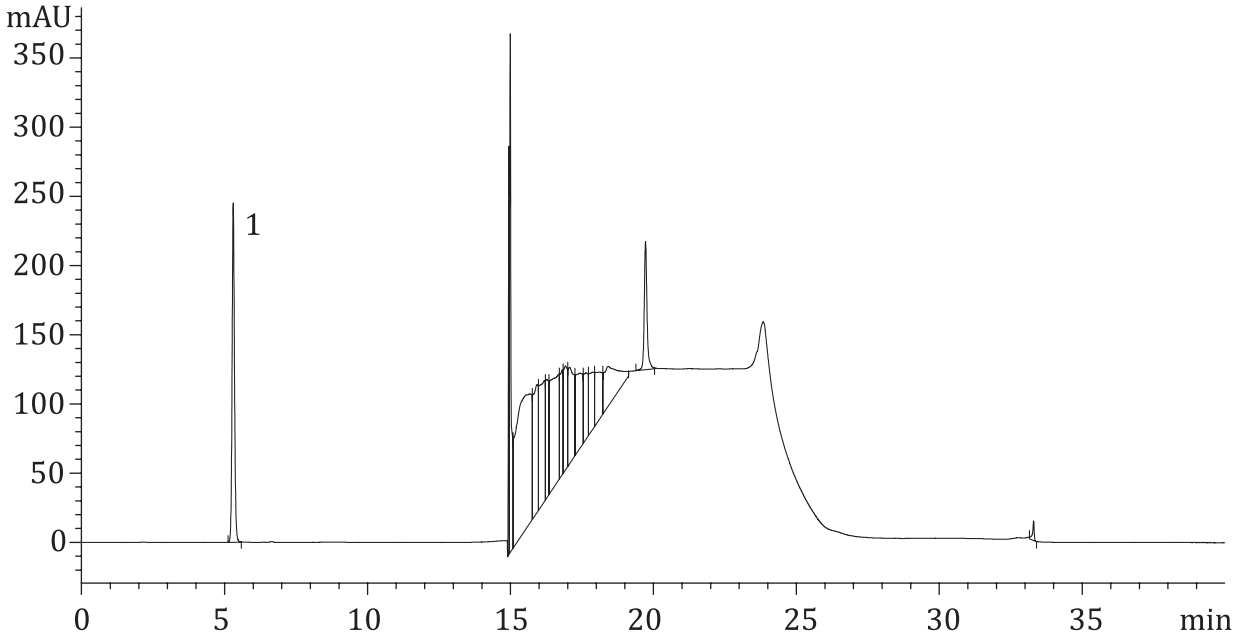
X theanine mg × l^{-1}

Y peak area (210 nm)

Figure A.1 — Standard curve

Annex B
(informative)

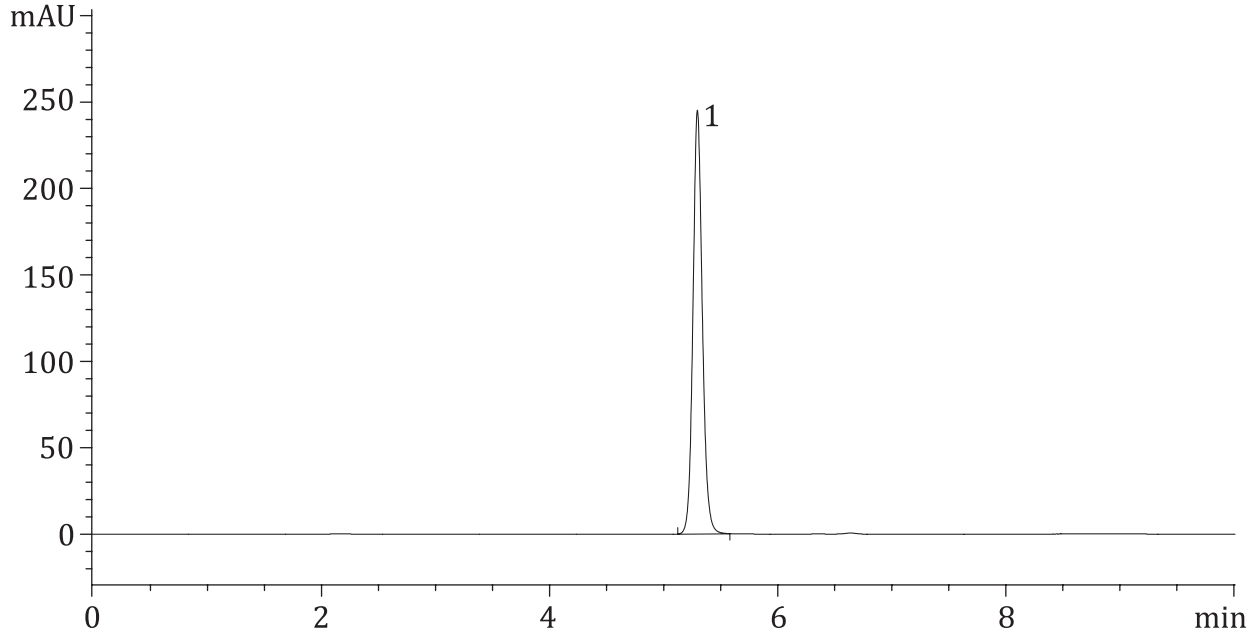
Typical chromatograms



Key

1 theanine retention time 5,298 min

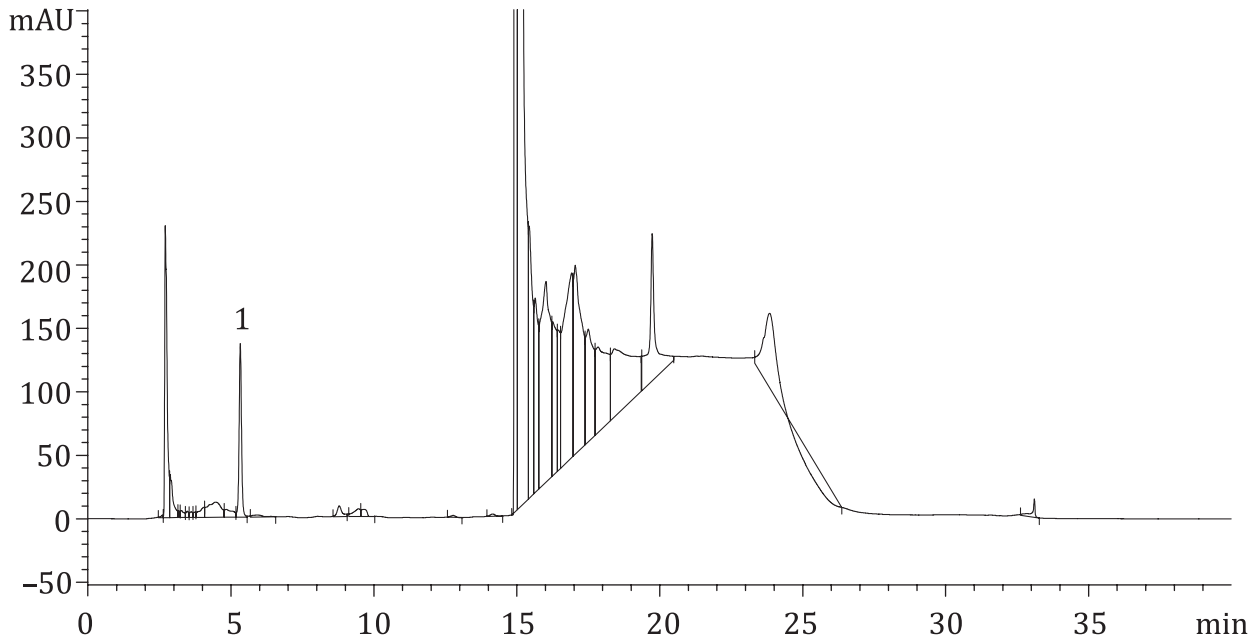
Figure B.1 — Full chromatogram of a theanine standard (100 µg/ml)



Key

1 theanine retention time 5,298 min

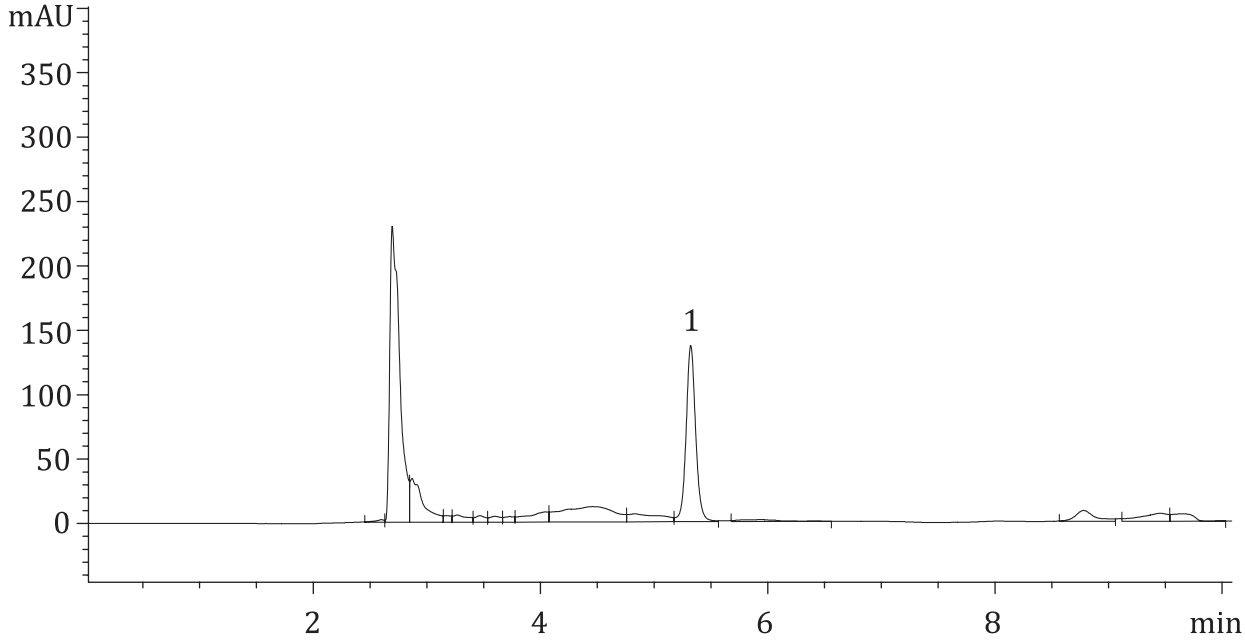
Figure B.2 — Extracted chromatogram of the theanine standard (100 µg/ml)



Key

1 theanine retention time 5,322 min

Figure B.3 — Full chromatogram of a black or green tea sample



Key

1 theanine retention time 5,322 min

Figure B.4 — Extracted chromatogram of a black or green tea sample

Annex C
(informative)

Results of interlaboratory tests

Interlaboratory tests, carried out during 2009/2012 under the auspices of the International Organization for Standardization, gave the statistical results (evaluated in accordance with ISO 5725-2) shown in Table C.1.

Table C.1 — Precision data

Sample	1	2	3	4	5
Number of participating laboratories	23	23	23	23	23
Number of accepted test results	23	23	22	23	22
Mean of theanine content, % w/w	0,595 9	0,538 7	0,777 3	3,189	0,601 1
Repeatability					
Standard deviation, s_r	0,021 7	0,018 1	0,016 7	0,051 3	0,014 2
Coefficient of variation, %	3,65	3,35	2,14	1,61	2,37
Limit, r	0,060 8	0,050 6	0,046 6	0,143 5	0,040 0
Reproducibility					
Standard deviation, s_R	0,060 7	0,043 70	0,046 1	0,255 0	0,085 5
Coefficient of variation, %	10,19	8,11	5,93	8,00	14,23
Limit, R	0,170 0	0,122 3	0,129 0	0,713 8	0,239 5
Key to samples: 1 Black tea from Assam, India. 2 Green tea from China. 3 Black tea from Kenya. 4 Green Gyokuro tea from Japan. 5 Black tea from Assam, India.					

Bibliography

[1] 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*

[2] Horanni R., & Engelhardt U.H. Enantiometric analysis of theanine in different teas (*Camellia sinensis*) using Marfey's agent. *Eur. Food Res. Technol.* 2015, **240** (1) pp. 61–70

[3] Ekborg-Ott K.H., Taylor A., Armstrong D.W. Varietal differences in the total and enantiomeric composition of theanine in tea. *J. Agric. Food Chem.* 1997, **45** pp. 353–363

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