Tentative Translation

# JAS 0002

JAPANESE AGRICULTURAL STANDARD

**Determination of the O-methylated Catechin in ‘Benifuuki’ Green Tea (*Camellia sinensis* L.)**
**—High-performance liquid chromatographic method**

2018 - 3 - 29

Ministry of Agriculture, Forestry and Fisheries

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Incorporated Administrative Agency
Food and Agricultural Materials Inspection Center

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(Tentative Translation) 0002 : 2018

# Determination of the O-methylated Catechin in 'Benifuuki' Green Tea (*Camellia sinensis* L.) — High-performance liquid chromatographic method

**Warning — Persons using this Standard should be familiar with normal laboratory practice. This standard does not purport to address all the safety problems associated with its use. It is the responsibility of the user of this standard to establish appropriate safety and health practices and to ensure compliance with any national regulatory conditions.**

## 1 Scope

This document specifies a high-performance liquid chromatographic method for the determination of (–)-epigallocatechin 3-(3"-O-methyl)gallate (EGCG3"Me), which is the one of methylated catechins, in the leaf tea of only 'Benifuuki' (*Camellia sinensis* var. *sinensis* cv. *Benifuuki*) green tea and/or those powder products.

## 2 Normative references

The following documents are referred to in the text in such a way that same or all of their content constitutes requirements of this document. For undated references, the latest edition of the referenced document (including any amendments) applies.

**ISO 565** Test sieves — Metal wire cloth, perforated metal plate and electroformed sheet — Nominal sizes of openings

**ISO 648** Laboratory glassware — Single-volume pipettes

**ISO 1042** Laboratory glassware — One-mark volumetric flasks

**ISO 3310-1** Test sieves — Technical requirements and testing — Part 1: Test sieves of metal wire cloth

**ISO 8655-2** Piston-operated volumetric apparatus — Part 2: Piston pipettes

**JIS K 0124** General rules for high performance liquid chromatography

**JIS K 0557** Water used for industrial water and wastewater analysis

**JIS K 8101** Ethanol (99.5)

**JIS K 8107** Disodium dihydrogen ethylenediamine tetraacetic acid dihydrate

**JIS K 9005** Phosphoric acid

**JIS K 9502** L(+)-Ascorbic acid (Reagent)

**JIS P 3801** Filter paper (for chemical analysis)

## 3 Principle

The EGCG3"Me content from a test portion of ground leaf tea is extracted with phosphoric acid/ethanol extraction mixture at 30 °C. The extract is filtered through a membrane filter. The EGCG3"Me in the extract are determined by high-performance liquid chromatograph (HPLC) using gradient elution with UV detection.

## 4 Reagents

Use only reagents recognized analytical grade, unless otherwise specified.

**Warning — It is the responsibility of users of this standard to comply with legal regulations regarding the use of reagents.**

**4.1 Water**, conforming to grade A3 or A4 of **JIS K 0557**.

**4.2 EGCG3"Me**, of minimum mass fraction, $\varphi(C_{23}H_{20}O_{11}) \geq 99$ % (HPLC).

**4.3 Phosphoric acid**, of minimum mass fraction, $\varphi(H_3PO_4) \geq 85\%$, according to **JIS K 9005**.

**4.4 Ethanol**, of minimum mass fraction, $\varphi(C_2H_5OH) \geq 99{,}5$ %, according to **JIS K 8101**.

**4.5 Methanol**, HPLC grade.

**4.6 Acetonitrile**, HPLC grade.

**4.7 L(+)-Ascorbic acid**, of minimum mass fraction, $\varphi(C_6H_8O_6) \geq 99{,}6\%$, according to **JIS K 9502**.

**4.8 Disodium dihydrogen ethylenediamine tetraacetate dehydrate** (EDTA2Na), of minimum mass fraction, $\varphi(C_{10}H_{14}N_2Na_2O_8 \cdot 2H_2O) \geq 99{,}5\%$, according to **JIS K 8107**.

**4.9 'Yabukita' Green Tea**, leaf tea or powder products.

**4.10 Phosphoric acid solution**, corresponding to approximately 2 %.

Mix 1 part per volume of phosphoric acid (**4.3**) with 49 parts per volume of water (**4.1**).

**4.11 Extraction solvent**, phosphoric acid/ethanol mixture

Mix equal volume of phosphoric acid solution (**4.10**) and ethanol (**4.4**).

**4.12 Dilution solvent**, 1,76 g/l of ascorbic acid solution containing 1,00 g/l of EDTA.

Dissolve 1,76 g of ascorbic acid (**4.7**) and 1,00 g of EDTA2Na (**4.8**) per 1000 ml of water (**4.1**).

**4.13 Blank samples extraction solution**, 'Yabukita' Green Tea extracts

Obtain the blank extract from 'Yabukita' Green Tea (**4.9**) in accordance with Clause **6** and **7.1**. Obtain the chromatograms of the blank extract in accordance with **7.3**, and confirm that the peak of EGCG3"Me is below the detection limit.

Transfer blank samples extraction solution into amber deactivated vials (**5.8**) labelled and store frozen. Immediately before use, thaw a frozen blank samples extraction solution by incubating at room temperature. Discard the remaining blank samples extraction solution, and do not refreeze.

**NOTE 1** The quantity or concentration of target component, which the ratio of signal (S) and noise (N), i.e., S/N value, is 3 can be taken as the limit of detection. The signal is the peak height that is the distance from the baseline to the top of EGCG3"Me peak at the chromatogram without noise, which is obtained by connecting the average values of detector output data with a line (see **JIS K 0124**). The noise is a half of the amplitude of difference of the maximum and minimum output signals in the baseline before and after the peak covering the range of 20 times the peak width at half height

on the chromatograms of the blank extract (see **JIS K 0124**).

**NOTE 2** It is confirmed that the blank samples extraction solutions will remain stable for at least 3 months when stored frozen at –25 °C or less.

**4.14 HPLC mobile phases**

**4.14.1 Mobile phase A**, approximately 0,2 % phosphoric acid solution

Mix 1 part per volume of phosphoric acid (**4.3**) with 420 parts per volume of water (**4.1**).

**NOTE** Appropriately degassing reduces the influence of dissolved gases in the mobile phase to HPLC determination.

**4.14.2 Mobile phase B**, methanol/acetonitrile mixture。

Mix 5 part per volume of acetonitrile (**4.6**) with 18 parts per volume of methanol (**4.5**).

**NOTE** Appropriately degassing reduces the influence of dissolved gases in the mobile phase to HPLC determination.

**4.15 EGCG3"Me stock standard solution**, corresponding to 100 μg/ml.

Weigh, to the nearest 0,01 mg, 2 mg or more of EGCG3"Me (**4.2**) into a volumetric flask (**5.3**) in order to prepare 100 μg/ml EGCG3"Me solution.

Add the dilution solvent (**4.12**) and dissolve sufficiently EGCG3"Me (e.g., by using ultrasonication ). Dilute to the mark with dilution solvent. Transfer stock standard solution into amber deactivated vials (**5.8**) labelled and store frozen.

**NOTE 1** Because EGCG3"Me in solutions are susceptible to degradation, it is important to add the EDTA and store into amber deactivated vials for the stock standard solution.

**NOTE 2** It is confirmed that the stock standard solutions will remain stable for at least 2 months when stored frozen at –25 °C or less.

Immediately before use, thaw a frozen stock standard solution by incubating at room temperature. Calculate the actual concentration (μg/ml) of EGCG3"Me in prepared stock standard solution.

**4.16 Dilute standard solutions**

**4.16.1 General**

Prepare dilute standard solutions of EGCG3"Me of 5 or more stepwise concentrations. Set the concentration which is included no other components interfering the determination of EGCG3"Me for highest level standard solution (see **7.3.2**). Prepare a series of the dilution standard solutions from a single vial of the EGCG3"Me stock standard solution (**4.15**). Prepare the solutions freshly on the day of use. Discard the remaining stock standard solution, and do not refreeze.

**NOTE** Because metal ion contamination of the chromatographic system appears to lead EGCG3"Me to degrade, it is important to add the blank samples extraction solution (**4.13**) for a series of the dilution standard solutions.

**4.16.2 A series of standard solutions**

Using a single-volume pipette (**5.6**) or piston pipette (**5.7**), transfer the EGCG3"Me stock standard solution (**4.15**), the dilution solvent (**4.12**) and the blank samples extraction solution (**4.13**) to an amber deactivated vial (**5.8**), and mix. Calculate the actual concentration (μg/ml) of EGCG3"Me in standard solutions, respectively.

**NOTE** The standard solutions, A, B, C, D and E prepared in the interlaboratory tests (**Annex A**) are described in **Table 1**.

**Table 1 — Preparation of standard solutions, A, B, C, D and E**

| Standard solutions | Volume taken from stock solution, µl | Volume taken from dilution solvent, µl | Volume taken from blank samples extraction solution, µl | Nominal concentration of EGCG3"Me standard solution, µg/ml |
|---|---|---|---|---|
| A | 500 | 400 | 100 | 50,0 |
| B | 250 | 650 | 100 | 25,0 |
| C | 100 | 800 | 100 | 10,0 |
| D | 50 | 850 | 100 | 5,00 |
| E | 10 | 890 | 100 | 1,00 |
| **NOTE** The values given are for guidance only. | | | | |

## 5 Apparatus

Usual laboratory apparatus and, in particular, the following.

**5.1 Analytical balances**, capable of weighing to an accuracy of ±1 mg and capable of weighing to an accuracy of ± 0.01 mg.

**5.2 Sieve**, of nominal size of the aperture 355 µm, ISO 3310-1.

**5.3 One-mark volumetric flasks**, to cover the volume range for standard and sample extract dilutions, and extraction, of **ISO 1042**, class A.

**5.4 Water bath**, capable of being maintained at (30 ± 3) °C.

**5.5 Membrane filters**, hydrophilic PTFE, of 0,45 µm pore size.

**5.6 Single-volume pipettes**, to cover the volume range for standard and sample extract dilutions, of **ISO 8655-2**, class A.

**5.7 Piston pipettes**, to cover the volume range for standard and sample extract dilutions, of **ISO 8655-2**, type A.

**5.8 Amber deactivated vials**, amber glass, of capacities 2 ml, for dilute standard solutions (see **4.17.1**).

**5.9 HPLC apparatus**

**5.9.1 High-performance liquid chromatograph**, equipped to perform binary gradient elution with a degassing unit, with a thermostatically controlled column compartment, an auto sampler, an ultraviolet detector set at 272 nm, and data collection/integration system, according to **JIS K 0124**. A photodiode array detector may be used instead of the UV detector.

**5.9.2 Chromatographic column for HPLC**, reverse-phase C18 (ODS) columns, with the following characteristics:

— length: 150 mm;

— internal diameter: 4,6 mm;

— spherical particle size: 5 µm.

— EGCG3"Me are eluted with no influence of other components within 12 min. Confirm retention time of EGCG3"Me in accordance with **7.3**.

If a guard column is used, select the guard column matching to the C18 (ODS) column.

## 6 Preparation of test samples

Grind the sample of leaf tea by using a suitable grinding device. Pass the ground material or powder products through a sieve (**5.2**). Proceed immediately in accordance with **7.1**, or store frozen the test samples.

**NOTE** It is confirmed that the ground samples will remain stable for at least 2 months when stored frozen at –25 °C or less.

Immediately before extraction (**7.1**), thaw a frozen test samples by incubating at room temperature.

## 7 Procedure

### 7.1 Extraction

Weigh, to nearest 1 mg, 240 mg to 260 mg of the test sample (Clause **6**) into a 25 ml one-mark volumetric flask (**5.3**). Add 20 ml of extraction solvent (**4.11**), and mix slightly. Place the volumetric flask containing the sample in the water bath (**5.4**) set at 30 °C, and allow 60 min for the extraction mixture to equilibrate. Remove the volumetric flask from the water bath, and allow it to cool to room temperature. Dilute to the mark with water (**4.1**), and mix.

Filter the mixture through a fluted filter paper (reject first filtered solution). Filter the filtered solution through a membrane filter (**5.5**) (reject first filtrate), and obtain approximately 1,5 ml of the filtrate. If stored frozen, immediately before dilution (**7.2**), thaw a sample extract by incubating at room temperature.

**NOTE** It is confirmed that the sample extracts will remain for at least 1 week if stored at –25 °C or less.

### 7.2 Dilution

Using a one-mark volumetric flask (**5.3**), a single-volume pipette (**5.6**) or piston pipette (**5.7**), dilute sample extract (**7.1**) with water (**4.1**). It is recommended to mix 1 part per volume of sample extract and 9 parts per volume of water. Transfer the sample extract dilution to an amber deactivated vial (**5.8**). Proceed immediately in accordance with **7.3**.

### 7.3 Determination

#### 7.3.1 HPLC operating conditions

Set up the chromatograph (**5.9**) in accordance with the manufacturer's instructions and adjust it as follows.

**a)** Flow rate of the mobile phase (**4.14**): 1,0 ml/min.

**b)** Temperature of the column (**5.9.2**) set at 40 °C

**c)** UV detector set at 272 nm.

**d)** Volume injected: 10 µl

**e)** Binary gradient conditions: 77 % mobile phase A (**4.14.1**) and 23 % mobile phase B (**4.14.2**) for 12 min, then increase the volume ratio of mobile phase B to elute quickly other analyte from the column. Then reset to 77 % mobile phase A and 23 % mobile phase B, and allow to equilibrate for 10 min before next injection.

**NOTE** The interlaboratory tests described in **Annex A** were performed by the binary gradient programme detailed in **Table 2**.

**Table 2 — Binary gradient programme**

| Time (min) | Mobile phase A (%) | Mobile phase B (%) |
|---|---|---|
| 0 to 12 | 77 | 23 |

| | | |
|---|---|---|
| 12 to 20 | 30 | 70 |
| 20 to 30 | 77 | 23 |
| **NOTE** The values given are for guidance only. | | |

**7.3.2 HPLC analysis**

Allow the entire system to run appropriately to stabilize it. Confirm that the fluctuation of base line gives no hindrance for determination of EGCG3"Me by a blank gradient run under the specified condition (**7.3.1**). Inject the standard solution with highest concentration (e.g., the standard solution A in **Table 1**) in a series of standard solutions (**4.16.2**) onto the column, and confirm that there are no peaks interfering with the determination of EGCG3"Me in the obtained chromatogram. Then inject a series of standard solutions onto the column, followed by the sample extract dilutions (**7.2**). It is recommended to repeat the injection of a certain standard solution (e.g., the standard solution C in **Table 1**) at regular intervals (typically after five test solutions).

**NOTE** The ratio of the largest peak area to the smallest peak area from the peak areas of EGCG3"Me in the repeated standard solution is normally 11/9 or less.

Collect data using the data collection/integration system for all peaks in the standards and test sample solutions.

**7.4 Identification**

Identify the individual EGCG3"Me by comparing retention times from sample chromatograms with those obtained from the standard solutions obtained under the same chromatographic conditions (**7.3.1**).

**NOTE** Typical HPLC chromatogram of a ‘Yabukita’ green tea and a ‘Benifuuki’ green tea can be found in **Annex B**.

## 8 Calculation

**8.1 General**

Quantitative determination is performed by the external standard method with integration of the peak area, which is then related to the corresponding value for the standard substance. In accordance with **JIS K 0124**, draw uniformly baselines for the peaks of EGCG3"Me in the standards and the samples in a HPLC analysis.

**8.2 Quantitation**

Construct linear calibration graphs for each standard from the EGCG3"Me concentrations (μg/ml) against the peak areas obtained from the data collection/integration system. The correlation coefficient of the linear calibration is required to be more than 0,995.

Calculate the concentration (μg/ml) of EGCG3"Me in the individual sample extract dilutions (**7.2**) by using the linear calibration. The EGCG3"Me content, $w_C$, expressed as a percentage by mass on a leaf tea sample or a power product, is given by the formula:

$$w_C = \frac{C \times V \times d \times 1000}{m \times 1000}$$

where

*C* is the EGCG3"Me concentration of the sample extract dilution, in μg/ml;

*V* is the extraction solvent volume, in milliliters, typically 25;

*d* is the dilution factor for the sample extract dilution (see **7.2**), typically 10;

*m* is the mass, in milligrams, of the sample test portion.

### 8.3 Expression of results

Express the results to two significant figures (e.g., 16 g/kg)

## 9 Precision

### 9.1 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 might not be applicable to concentration ranges (11 g/kg to 19 g/kg) and matrices other than those given.

### 9.2 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 be expect in not more than 5 % of cases be greater than the repeatability limit ($r$) values[1] given in **Table A.1**.

### 9.3 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 be expect in not more than 5 % of cases be greater than the reproducibility limit ($R$) values[1] given in **Table A.1**.

## 10 Quality control

The laboratory is required to have internal quality control procedures for tests.

## 11 Test report

The test report shall include at least the following information:

**a)** a reference to this JAS standard;

**b)** identification of the sample;

**c)** the data of the test;

**d)** the results of the test;

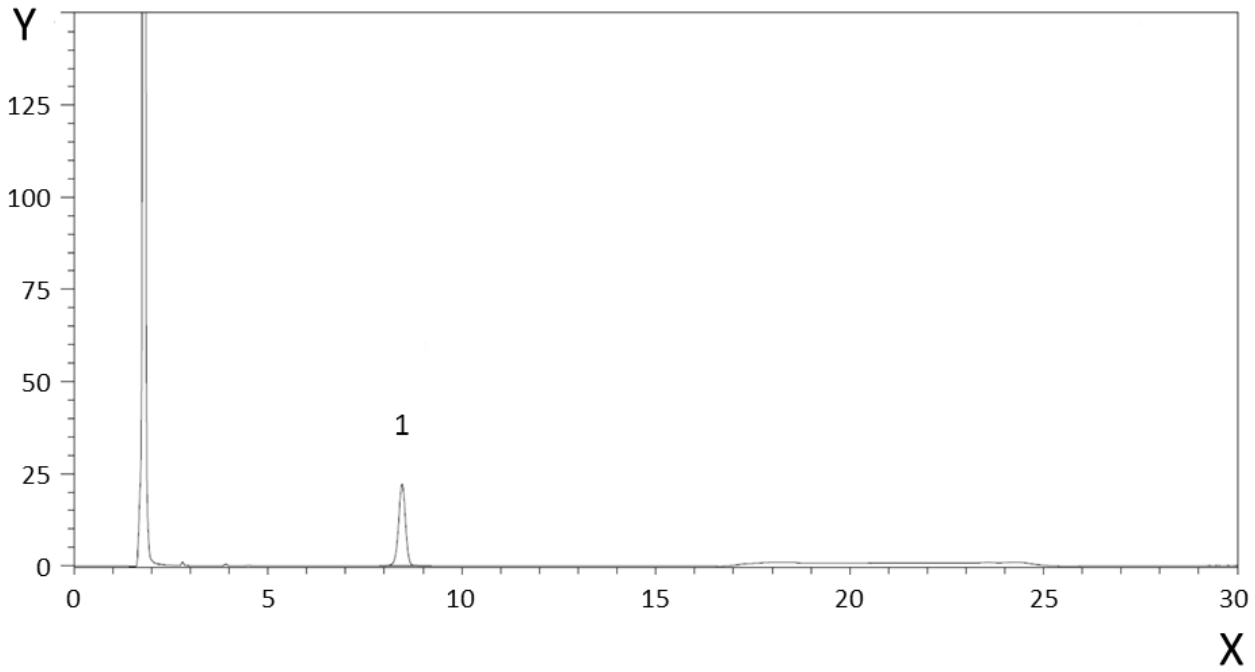
# Annex A
# (informative)
# Results of interlaboratory tests

Interlaboratory tests in accordance with IUPAC protocol[2] carried out in 2015 in Japan, gave the statistical results shown in **Tables A.1**[3]. The homogenous[4] samples were prepared from a commercial 'Benifuuki' green leaf tea and commercial powder products of 'Benifuuki' green tea. A stock standard solution of known EGCG3"Me concentration and a blank samples extraction solution as well as the experimental protocol and test samples were supplied to the participants by the Food and Agricultural Materials Inspection Center (FAMIC) organized this interlaboratory tests. All participants, respectively, tested a total of 10 test samples (5 pairs of blind duplicates) according to the experimental protocol.

**Table A.1 — Precision data**

| Sample identification | Sample 1 (leaf tea) | Sample 2 (powder product) | Sample 3 (powder product) | Sample 4 (powder product) | Sample 5 (powder product) |
|---|---|---|---|---|---|
| Number of participating laboratories | 10 | 10 | 10 | 10 | 10 |
| Number of accepted test results | 10 | 8 | 10 | 8 | 10 |
| Mean EGCG3"Me content, g/kg | 10,85 | 10,77 | 13,65 | 15,59 | 18,89 |
| Repeatability standard deviation $s_r$, g/kg | 0,22 | 0,15 | 0,20 | 0,21 | 0,30 |
| Repeatability relative standard deviation, % | 2,0 | 1,4 | 1,5 | 1,4 | 1,6 |
| Repeatability limit $r$ ($r = 2{,}8\ s_r$) , g/kg | 0,61 | 0,42 | 0,57 | 0,59 | 0,84 |
| Reproducibility standard deviation $s_R$, g/kg | 0,62 | 0,17 | 0,54 | 0,25 | 0,76 |
| Reproducibility relative standard deviation, % | 5,7 | 1,6 | 4,0 | 1,6 | 4,0 |
| Reproducibility limit $R$ ($R = 2{,}8\ s_R$), g/kg | 1,7 | 0,47 | 1,5 | 0,71 | 2,1 |

# Annex B
# (informative)
# Typical HPLC chromatograms




**Key**

X Retention time, min

Y Response, mAU

1 EGCG3"Me

**Figure B.1 — EGCG3"Me solution**

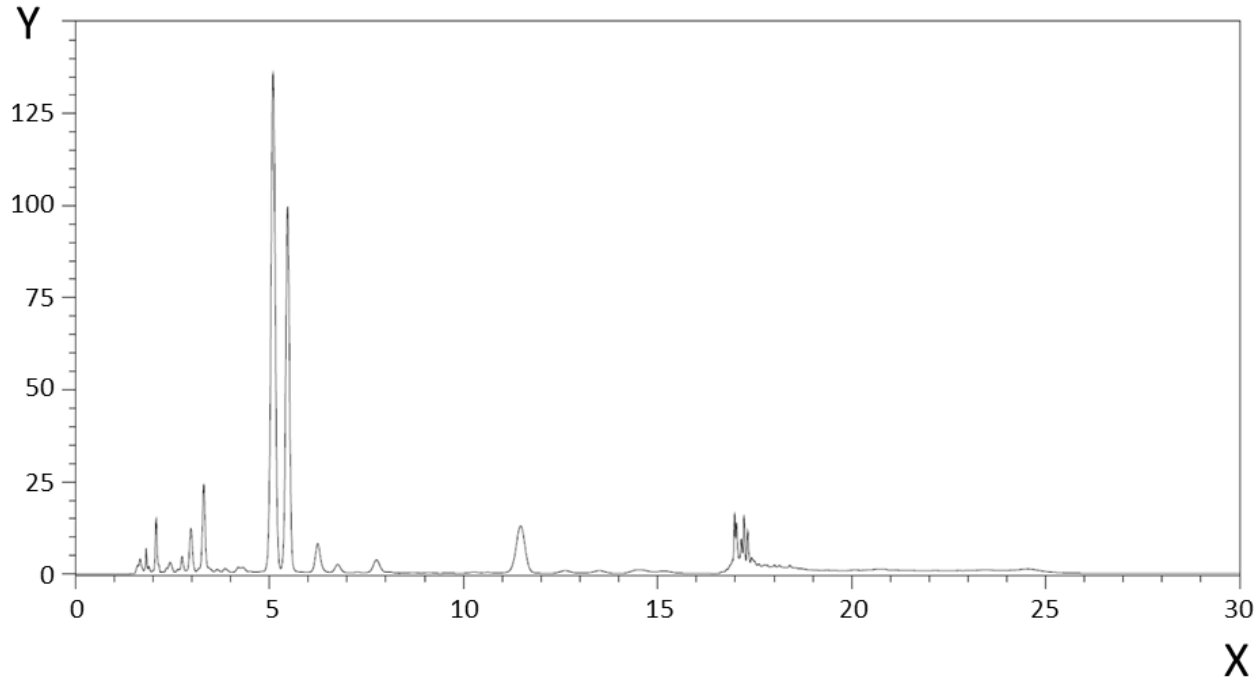
**HPLC operating conditions**

HPLC operating conditions in accordance with **7.3.1** and the following.

a) Chromatographic column : Wakopak® Navi C18-5[1)]

b) Binary gradient programme : **Table 2**

1) Wakopak® is an example of a suitable product available commercially. This information is given for the convenience of users of this standard and does not constitute an endorsement of this product by Ministry of Agriculture, Forestry and Fisheries.




**Key**

X Retention time, min

Y Response, mAU

**Figure B.2 — 'Yabukita' Green Tea extract**

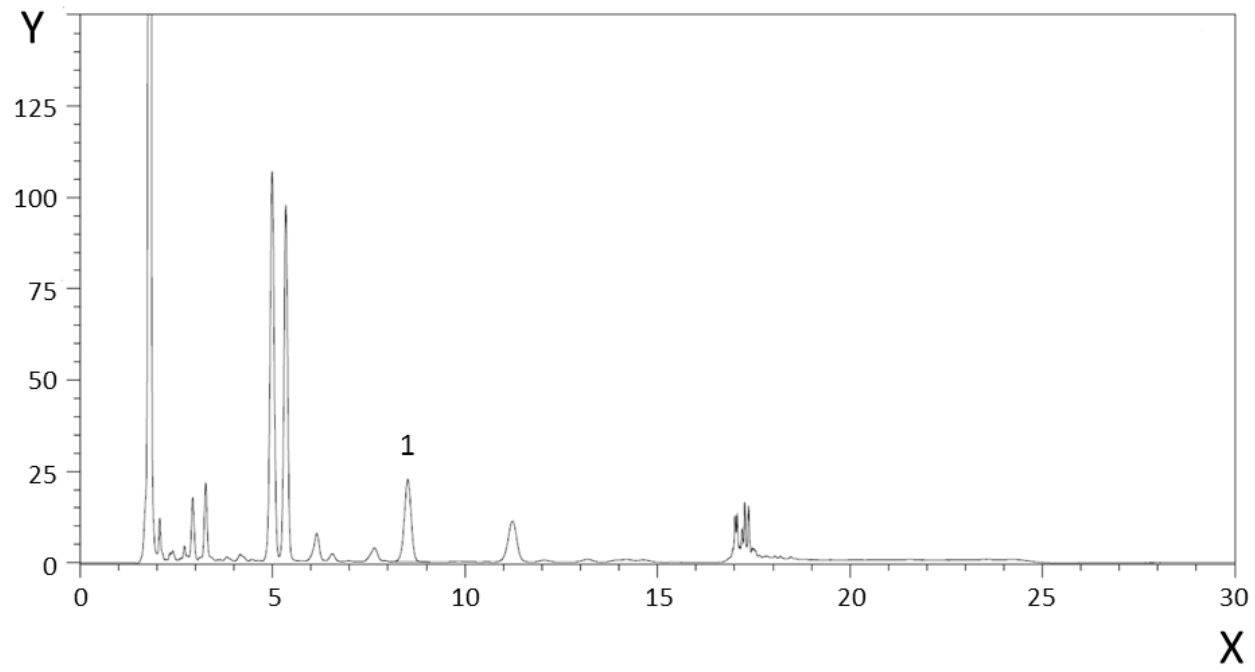
**HPLC operating conditions**

HPLC operating conditions in accordance with **7.3.1** and the following.

a) Chromatographic column : Wakopak® Navi C18-5[2)]

b) Binary gradient programme : **Table 2**

2) Wakopak® is an example of a suitable product available commercially. This information is given for the convenience of users of this standard and does not constitute an endorsement of this product by Ministry of Agriculture, Forestry and Fisheries.




**Key**

X Retention time, min

Y Response, mAU

1 EGCG3"Me

**Figure B.3 — EGCG3"Me standard solution, containing blank samples extraction solution**

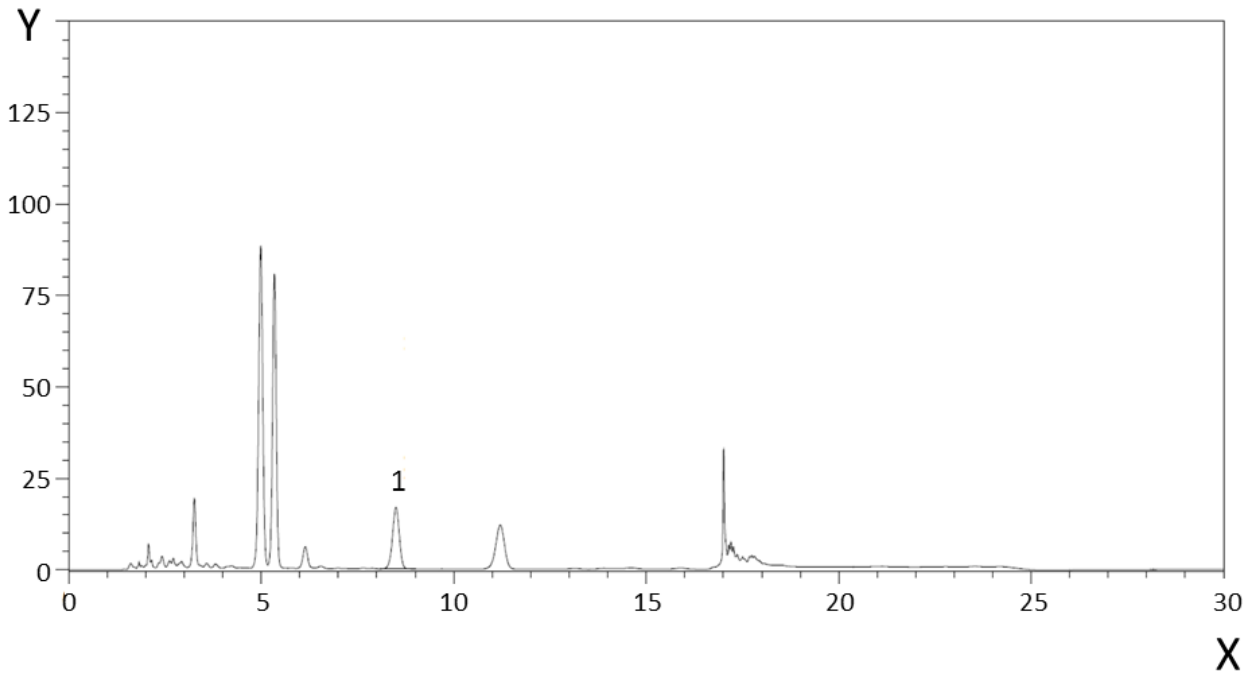
**HPLC operating conditions**

HPLC operating conditions in accordance with **7.3.1** and the following.

a) Chromatographic column : Wakopak® Navi C18-5[3)]

b) Binary gradient programme : **Table 2**

3) Wakopak® is an example of a suitable product available commercially. This information is given for the convenience of users of this standard and does not constitute an endorsement of this product by Ministry of Agriculture, Forestry and Fisheries.




**Key**

X Retention time, min

Y Response, mAU

1 EGCG3"Me

**Figure B.4 — 'Benifuuki' green tea extract**

**HPLC operating conditions**

HPLC operating conditions in accordance with **7.3.1** and the following.

a) Chromatographic column : Wakopak® Navi C18-5[4)]

b) Binary gradient programme : **Table 2**

4) Wakopak® is an example of a suitable product available commercially. This information is given for the convenience of users of this standard and does not constitute an endorsement of this product by Ministry of Agriculture, Forestry and Fisheries.

# Bibliography

[1] **ISO 5725** (all parts) Accuracy (trueness and precision) of measurement methods and results

[2] Horwitz, W., Protocol for the design, conduct and interpretation of method-performance studies, *Pure* & *Appl. Chem.*, 1995, **67**(2), p. 331–343

[3] Homura, Y., Hiejima, Y., Kodama, T., Tanaka, M., Horie, H, Suzuki, T., Yasui, A., Validation of Method for Determining *O*-methylatedCatechin in ‘Benifuuki’ Green Tea (*Camellia sinensis* L.) by Interlaboratory Study, *Nippon Shokuhin Kagaku Kogaku Kaishi*, 2016, **63**(7), p. 312–318.

[4] Naito, S., Tsukkoshi, Y., De-ta no Toukeiteki Toriatsukai, *Shokuryo*, 2008, **46**, p. 27–62.