Tentative Translation

# JAS 0008

JAPANESE AGRICULTURAL STANDARD

**Determination of the lutein in Spinach**
**—High-performance liquid chromatographic method**

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Ministry of Agriculture, Forestry and Fisheries

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Incorporated Administrative Agency
Food and Agricultural Materials Inspection Center

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# Japanese Agricultural Standard

JAS
0008：2019

## Determination of the lutein in Spinach
## －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 test method of lutein in the edible part of spinach (*Spinacia oleracea* L.)(fresh spinach and frozen one after blanching).

### 2 Normative references

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

**ISO 648** Laboratory glassware — Single-volume pipettes
**ISO 1042** Laboratory glassware — One-mark volumetric flasks
**ISO 8655-2** Piston-operated volumetric apparatus — Part2:Piston pipettes
**JIS K 0115** General rules for molecular absorptiometric analysis
**JIS K 0124** General rules for high performance liquid chromatography
**JIS K 0557** Water used for industrial water and wastewater analysis
**JIS K 8034** Acetone
**JIS K 8101** Ethanol(99,5)
**JIS K 8150** Sodium chloride
**JIS K 8359** Ammonium acetate
**JIS K 8361** Ethyl acetate (Reagent)
**JIS K 8574** Potassium hydroxide
**JIS K 8780** Pyrogallol
**JIS K 8848** Hexane
**JIS K 8891** Methanol
**JIS K 8987** Sodium sulfate

### 3 Principle

The test portion of ground spinach which is homogenized with pyrogallol ethanol is saponified with potassium hydroxide. The lutein is extracted by hexane and ethyl acetate mixture.
The lutein in the extract is determined by high-performance liquid chromatograph (HPLC) with ultraviolet-visible absorption detector.

## 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 Lutein,** of minimum mass fraction, $\varphi$ ($C_{40}H_{56}O_2$) ≥ 95,0%.

**4.3 Ethanol,** of minimum mass fraction, $\varphi$($C_2H_5OH$) ≥ 99,5 %, according to **JIS K 8101**.

**4.4 Pyrogallol,** of minimum mass fraction, $\varphi$($C_6H_6O_3$) ≥ 99,0 %, according to **JIS K 8780**.

**4.5 Potassium hydroxide,** of minimum mass fraction, $\varphi$(KOH) ≥ 85,0 %, according to **JIS K 8574**.

**4.6 Sodium chloride,** of minimum mass fraction, $\varphi$(NaCl) ≥ 99,5 %, according to **JIS K 8150**.

**4.7 Hexane ,** of minimum mass fraction, $\varphi$($CH_3$ $(CH_2)_4CH_3$) ≥ 96,0 %, according to **JIS K 8848**.

**4.8 Ethyl acetate,** of minimum mass fraction, $\varphi$($CH_3COOC_2H_5$) ≥ 99,5 %, according to **JIS K 8361**.

**4.9 Methanol,** HPLC grade.

**4.10 Acetonitrile,** HPLC grade.

**4.11 Ethanol(HPLC),** HPLC grade.

**4.12 Ammonium acetate,** according to **JIS K 8359**.

**4.13 Nitrogen,** of volume fraction, ($N_2$) ≥99,5%.

**4.14 2,6-di-*tert*-butyl-*p*-cresol(BHT),** of minimum mass fraction, $\varphi$ ($C_6H_2$ $(OH)(C(CH_3)_3)_2CH_3$) ≥98%.

**4.15 Pyrogallol ethanol,**

Dissolve 30 g of pyrogallol (**4.4**) per 1,0 l of ethanol (**4.3**). Prepare it when necessary.

**4.16 Potassium hydroxide (water) solution,**

Dissolve 60 g of potassium hydroxide (**4.5**) per 100 ml of water (**4.1**).

**Warning— Since irritating gas is generated, work should be done in a place with good ventilation inside a fume cupboard etc.**

**4.17 Sodium chloride (water) solution,**

Dissolve 10 g of sodium chloride (**4.6**) per 1,0 l of water (**4.1**).

**4.18 Hexane / ethyl acetate mixture,**

Mix 9 parts per volume of hexane (**4.7**) with 1 part per volume of ethyl acetate (**4.8**).

**4.19 BHT ethanol,**

Dissolve 1,0 g of BHT (**4.14**) per 1,0 l of ethanol (**4.11**).

**4.20 HPLC mobile phases**

**4.20.1 Mobile phase A**, acetnitrile / methanol mixture containing ammonium acetate.

Dissolve 5,0g of ammonium acetate (4.12) per 1,0 l of methanol (**4.10**). Mix 4 parts per volume of this solution with 15 parts per volume of acetonitrile. Degas the mixture before use.

**NOTE** Degassing prevents troubles of gases, then gives stable flow and background.

**4.20.2 Mobile phase B**, ethanol.

Degas ethanol (**4.11**) before use.

**NOTE** Degassing prevents troubles of bubbles, then improves baseline stability and reduce noise.

**4. 21 Lutein stock standard solution,**

Prepare a solution containing lutein (**4.2**) in ethanol (**4.11**) at a concentration of 100 μg /ml. Transfer this solution into a labelled bottle with screw cap, then store at –20 °C or lower under light-shielded condition.

**NOTE** Lutein stock standard solution is sometimes degraded gradually.

Remove from freezer before use, allow it to attain to room temperature and mix.

**4.22 Standard solutions**

**4.22.1 General**

Prepare lutein standard solutions of 4 or more stepwise concentrations.

A standard solution for absorbance measurement (4.22.2) and a series of the following standard solutions (4.22.3) should be prepared from a single bottle of the lutein stock standard solution (**4.21**). Every time each lutein stock standard solution is returned to normal temperature, prepare the standard solution for absorbance measurement and measure the concentration of that on the same day. Discard the remaining stock standard solution after used, and do not reserve.

**4.22.2 Standard solution for absorbance measurement**,

Dilute the lutein stock standard solution (**4.21**) 50-fold with ethanol (**4.3**) using the single volume pipette (**5.6**) or the piston pipette (**5.7**) and volumetric flask (**5.5**). This solution is used for absorbance measurement.

**NOTE** In the interlaboratory tests described in Annex A, 0,200 ml of lutein stock standard solution was transferred into a 10 ml volumetric flask using the piston pipette.

Set up and operate the spectrometer (**5.14**) in accordance with the manufacturer's instructions.

Measure the concentration of standard solution for absorbance measurement by using a spectrometer at 445 nm with ethanol as reference.

The lutein concentration of the stock standard solution in μg/ml, ρ, is given by the formula:

$$\rho = \frac{A \times V_1 \times 10\,000}{\varepsilon \times V_2}$$

where

$A$ is the absorbance of standard solution for absorbance measurement determined at 445 nm (ethanol ,1 cm cell );

$\varepsilon$ is the absorption coefficient of lutein in concentration 1% and optical pathlength 1 cm, 2 550[5];

$V_1$ is used volumetric flask's capacity, in ml;

$V_2$ is the volume, in ml, of used stock standard solution;

**Note** In the interlaboratory tests described in Annex A, $V_1$ is 10 and $V_2$ is 0,200.

After calculation, proceed immediately in accordance with **4.22.3**.

**4.22.3 A series of standard solution**

Using a single volume pipette(**5.6**) or a piston pipette (**5.7**), transfer lutein stock standard solution (**4.21**) into glass vessels (**5.9**).

Evaporate the solutions just to dryness under a stream of nitrogen gently.

Dissolve the contents in glass vessels completely by BHT ethanol (**4.19**).

**Note1** In the interlaboratory tests described in Annex A, test tube mixier was used for about 10 seconds.

Transfer the dilute standard solutions into vials (**5.13**).

The lutein concentration of the dilute standard solution in μg/ml, $\rho_i$, is given by the formula:

$$\rho_i = \frac{\rho \times V_3}{V_4}$$

where

$\rho$ is the concentration of lutein stock standard solution (**4.21**), in μg/ml;

$V_3$ is the volume of lutein stock standard solution (**4.21**) , in ml,;

$V_4$ is the volume of BHT ethanol (**4.19**), in ml;

**NOTE2** In the interlaboratory tests described in **Annex A**, each lutein standard solutions were prepared 1,0, 2,0, 5,0, 10 and 20 μg/ml concentration.

Perform HPLC analysis (**7.4.2**) on the day of preparation.

## 5 Apparatus

Usual laboratory apparatus and, in particular, the following.

**5.1 Analytical balances**, capable of weighing to an accuracy of ±0,1mg more than 200 g.

**5.2 Centrifuge tubes,** glass or polypropylene, of about 50 ml capacity, with the lid, keeping the space for enough mix and centrifugation at 400×*g* is possible.

Use the stopper or screw type lid to which a packing of a material resistant to organic solvents and strong basic solutions is attached.

**5.3 Vertical Shaker,** for bobbing or back-and-forth motion holding tube.

**5.4 Centrifuge,** capable of 400×*g*.

**Warning — To prevent accidents, operate the centrifuge according to the instruction manual of the equipment.**

**5.5 One-mark volumetric flasks**, to cover the volume range for standard dilution and sample extract dissolution, of **ISO1042**, class A.

**5.6 Single volume pipettes,** to cover the volume range for standard dilutions, of **ISO648**, class A.

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

**5.8** **Water bath**, capable of being maintained at (70±3) °C and size for centrifuge tube rack.

**5.9** **Glass vessels**, amber, to cover the volume range for lutein stock standard dilutions.

**5.10** **Round-bottomed flasks,** of 100 ml capacity, with ground neck and amber, usable evaporation.

**5.11** Rotary **evaporator,** with water bath and vacuum control, for evaporation of solvent, for example hexane, ethyl acetate and ethanol.

**5.12** **Membrane filters**, for organic solutions, made of polytetrafluoroethylene (PTFE), with a pore size of less than 0,20 µm. The filter and the housing are unitary, and the material of the housing is resistant to organic solvents.

**5.13** **Vials**, suitable for HPLC to be used. Amber and deactivated glass, with deactivated insert vials in them, or other glass-made ones that have been checked for no influence. The septum of the lid was coated with PTFE or PTFE.

**5.14** **Spectrometer,** capable measuring wavelengths 445 nm, holding cells (**5.15**).

**5.15** **Cells,** quartz glass or glass, of optical path length 1 cm, and they should have stoppers. When multiple cells are used, use the ones that guaranteed to have same optical characteristic.

**5.16** **HPLC apparatus**

**5.16.1** **High-performance liquid chromatograph**, equipped to perform binary gradient elution or switching solvent line manually with a thermostatically controlled column compartment, an ultraviolet-visible detector set at 445 nm, and data collection / integration system.

**5.16.2** **Chromatographic column for HPLC**, reverse-phase C30 (Triacontyl) columns, with the following characteristics:—length: 250 mm;

—internal diameter: 4,6 mm;

—spherical particle size: 5 µm

Lutein shall elute within 15 min. Confirm that retention time of the lutein and the peak of lutein does not overlap with those of other peaks according to **7.**4.

When a guard column is used, select the one matching to the C30 (Triacontyl) column.

## 6 Preparation of test samples

**6.1** Mince the sample of fresh spinach removed the roots and weigh it in the container like a homogenizer cup. When the sample is frozen spinach that is only blanched, weigh it in the container. Record the weight in 3 significant figures.

**6.2** Add pyrogallol ethanol (**4.15**) about 3 times mass of sample (**6.1**) and weigh it in 3 significant figures.

**6.3** Pulverize it using homogenizer or the like. This is used as a test sample.

**6.4** Proceed immediately in accordance with **7.1**, or store frozen the test samples at –20 °C or lower.

If test samples are stored at –20 °C or lower, transfer all of them or a portion of them after stirring homogeneously into the amber glass sealed containers soon after pulverized. Remove the test samples stored at –20 °C or lower from the freezer before use, allow them to room temperature and mix well.

**NOTE** It has been confirmed that the test samples will remain stable for at least 6 month when stored frozen at –20 °C to –30 °C.

## 7 Procedure

### 7.1 Saponification

**7.1.1** Weigh, to the nearest 10 mg, approximately 2 g of the test sample (**6**) into a tube (**5.2**). Add 10 ml of pyrogallol ethanol (**4.15**) to the tube.

**7.1.2** Add 1 ml of potassium hydroxide (water) solution (**4.16**) to a tube, mix gently. Place the tube in a constant temperature water bath (**5.8**) set at 70 °C, heat the tube for 30 min mixing every 5 min. Allow the centrifuge tube to cool to room temperature.

### 7.2 Extraction of lutein

**7.2.1** Add 20 ml of sodium chloride (water) solution (**4.17**) and 12 ml of hexane / ethyl acetate mixture (**4.18**) to a tube (**7.1.2**) and mix.

**7.2.2** Mix hard the contents for 5 min by vertical shaker (**5.3**).

Separate the contents by the centrifuge(**5.4**) at 400×*g* for 5 min. Transfer the supernatant into a round-bottomed flask (**5.10**).

**7.2.3** Add 12 ml of hexane / ethyl acetate mixture to the liquid left in the tube. Repeat steps **7.2.2**. Transfer the supernatant in the same round-bottomed flask which the **7.2** supernatant was transferred.

**7.2.4** Repeat steps **7.2.3**.

**7.2.5** Evaporate almost the solvent in a round-bottomed flask (**7.2.4**) at less than 40 °C by vacuum rotating evaporator (**5.11**). Then evaporate the solvent under a gentle stream of nitrogen (**4.13**).

### 7.3 Dissolution

Dissolve the contents of the round-bottom flask (**7.2.5**) completely, using BHT ethanol (**4.19**). Then transfer the solution completely to a volumetric flask.

**NOTE 1** 10 ml of one-mark volumetric flasks were used in the interlaboratory tests described in **Annex A.**

Add to the mark with BHT ethanol and mix. Filter through a membrane filter (**5.12**) and transfer filtrate into the vials (**5.13**). This filtrate is used as sample extract.

Perform HPLC measurement (**7.4.2**) on the day of preparation or store the sample extract at –20 °C or lower.

**NOTE 2** It has been confirmed that the sample extract will remain stable for at least 12 days when stored at–30 °C to –20 °C.

Remove the stored sample extract stored at –30 °C to –20 °C from the frozen before use and mix well.

### 7.4 Determination

#### 7.4.1 HPLC operating conditions

Set up the chromatograph (**5.16**) in accordance with the manufacturer's instructions and adjust it as follows.

a) Flow rate of the mobile phase (**4.20**): 1,0 ml/min

b) Temperature of the column (**5.16.2**) set at 40 °C

c) UV-VIS detector set at 445 nm.

d) Volume injected: 10 µl

e) Binary gradient conditions: 95 % mobile phase A (**4.20.1**) and 5 % mobile phase B (**4.20.2**) for 15 min from injections of sample extract, then increase the volume ratio of mobile phase B to 95 % in order to elute quickly other analyte from the column.

Then reset to 95 % mobile phase A and 5 % mobile phase B, and allow to equilibrate for 10 min before next injection. If a lutein peak is not disturbed by those of other components, the washing time , equilibrate time and volume ratio of solvents may be changed.

**NOTE** In the interlaboratory tests described in Annex A, the elution programme as detailed in **Table 1** or manual switching solvent lines with solvents that were mixed as table 1 beforehand were used.

**Table 1 — Binary gradient programme**

| Time (min) | Mobile phase A (%) | Mobile phase B (%) |
|---|---|---|
| 0 to 15 | 95 | 5 |
| 15 to 30 | 5 | 95 |
| 30 to 40 | 95 | 5 |
| **NOTE** The values given are for guidance only. | | |

### 7.4.2 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 lutein by a blank run under the specified condition (**7.4.1**). Then inject a series of standard solutions (**4.22.3**) onto the column, followed by an equal volume of the sample extract solutions (**7.3**).

### 7.4.3 Identification

Identify the individual lutein peak in the sample chromatograph by comparing retention times with those obtained from the standard solutions under the same chromatographic conditions (**7.4.1**).

**NOTE** Typical HPLC chromatogram of a 'Spinach' extract 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.

For the peaks of impurities, take appropriate measures according to the perpendicular or tangent method prescribed by **JIS K 0124**.

### 8.2 Quantitation

Obtain the areas of lutein in each of a series of standard solutions (**4.22.4**). Construct linear calibration graphs for each standard of the lutein 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 lutein from the areas of each sample solutions by the calibration curve. The lutein content, $W_i$, expressed as a percentage by mass on 'Spinach' sample, is given by the formula:

$$W_i = \frac{C \times V_5}{W \times \dfrac{W_{sp}}{W_{sp} + W_{et}}}$$

where

$C$ is the concentration of lutein in the the diluted sample extract, in µg/ml;

$V_5$ is the constant volume (ml) at the time of dissolution, in the interlaboratory tests, that is, 10;

$W$ is the mass, in g, of the sample test portion(**7.1.1**);

$W_{sp}$ is the mass, in g, of the sample spinach in preparation (**6.1**);

$W_{et}$ is the mass, in g, of the pyrogallol ethanol in preparation(**6.2**);

**NOTE** In the interlaboratory tests described in Annex A, $V_5$ is 10.

### 8.3 Expression of results

Express the results to two significant figures

## 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 (65 mg/kg to $1{,}5 \times 10^2$ mg/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 expected in not more than 5 % of cases be greater than the repeatability limit ($r$) values[2] given in Table A.1 on average as long as the specified operation is definitely done[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 expected in not more than 5 % of cases be greater than the reproducibility limit ($R$) values[2] given in Table A.1 on average as long as the specified operation is definitely done[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 date of the test;

d) the results of the test.

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

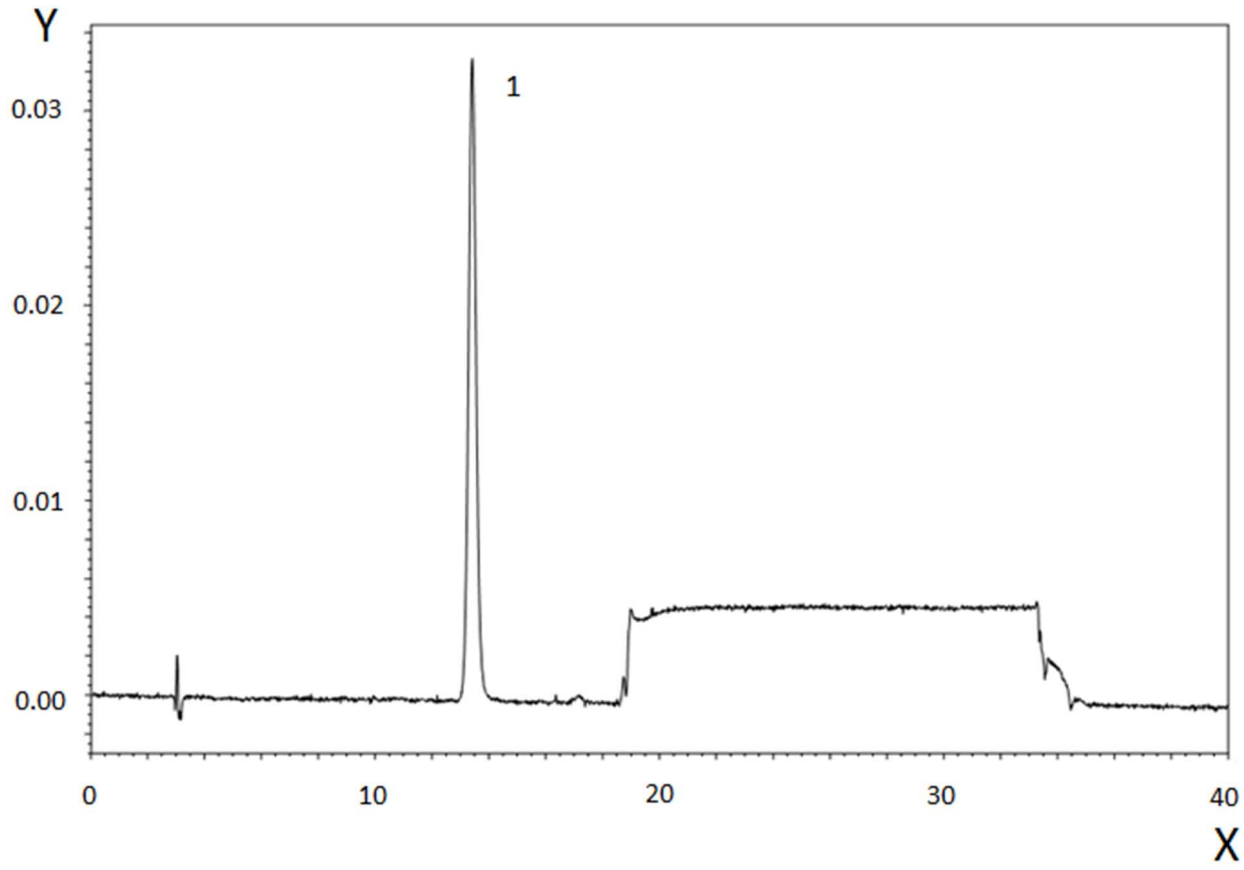
Interlaboratory tests in accordance with IUPAC protocol[3], carried out in 2017 in Japan, gave the statistical results shown in Tables A.1. Some samples from which the root tops of commercially available '*Spinicia oleracea* L.' was removed, 50 g, were added 30 g/L pyrogallol ethanol with 3 times of sample mass as an antioxidant, and were grinded at 5 000 r/min for 5 min by homogenizer.

After homogeneity [4] was confirmed, pulverized samples were used as a test sample. Since lutein may be easily degraded, it was used after purified as described in Annex C. The experimental protocol, standard stock solutions and test samples were supplied to the participating laboratories by the Food and Agricultural Materials Inspection Center (FAMIC). All laboratories, 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 | Sample 2 | Sample 3 | Sample 4 | Sample 5 |
|---|---|---|---|---|---|
| Number of participating laboratories | 12 | 12 | 12 | 12 | 12 |
| Number of accepted test results | 10 | 11 | 12 | 12 | 12 |
| Mean lutein content, mg/kg (mass fraction), | 64,9 | 71,8 | 88,8 | 119 | 150 |
| Repeatability standard deviation $s_r$, mg/kg | 3,3 | 3,6 | 6,7 | 4,8 | 5,1 |
| Repeatability relative standard deviation, % | 5,1 | 5,0 | 7,5 | 4,1 | 3,4 |
| Repeatability limit $r$ ($r$ = 2,8 $s_r$), mg/kg | 9,2 | 10 | 19 | 13 | 14 |
| Reproducibility standard deviation $s_R$, mg/kg | 3,6 | 5,4 | 12 | 7,5 | 6,8 |
| Reproducibility relative standard deviation, % | 5,6 | 7,5 | 13 | 6,4 | 4,6 |
| Reproducibility limit $R$ ($R$ = 2,8 $s_R$), mg/kg | 10 | 15 | 33 | 21 | 19 |

# Annex B
# (informative)
# Typical HPLC chromatograms [1)]




**Key**

X Retention time, min

Y Response, AU

1 Lutein

**FigureB.1 − Lutein standard**

**HPLC Condition**

HPLC operating conditions in accordance with **7.4.1** and the following.

a) Chromatographic column : YMC® Carotenoid [2)]

b) Binary gradient conditions: **Table 1**

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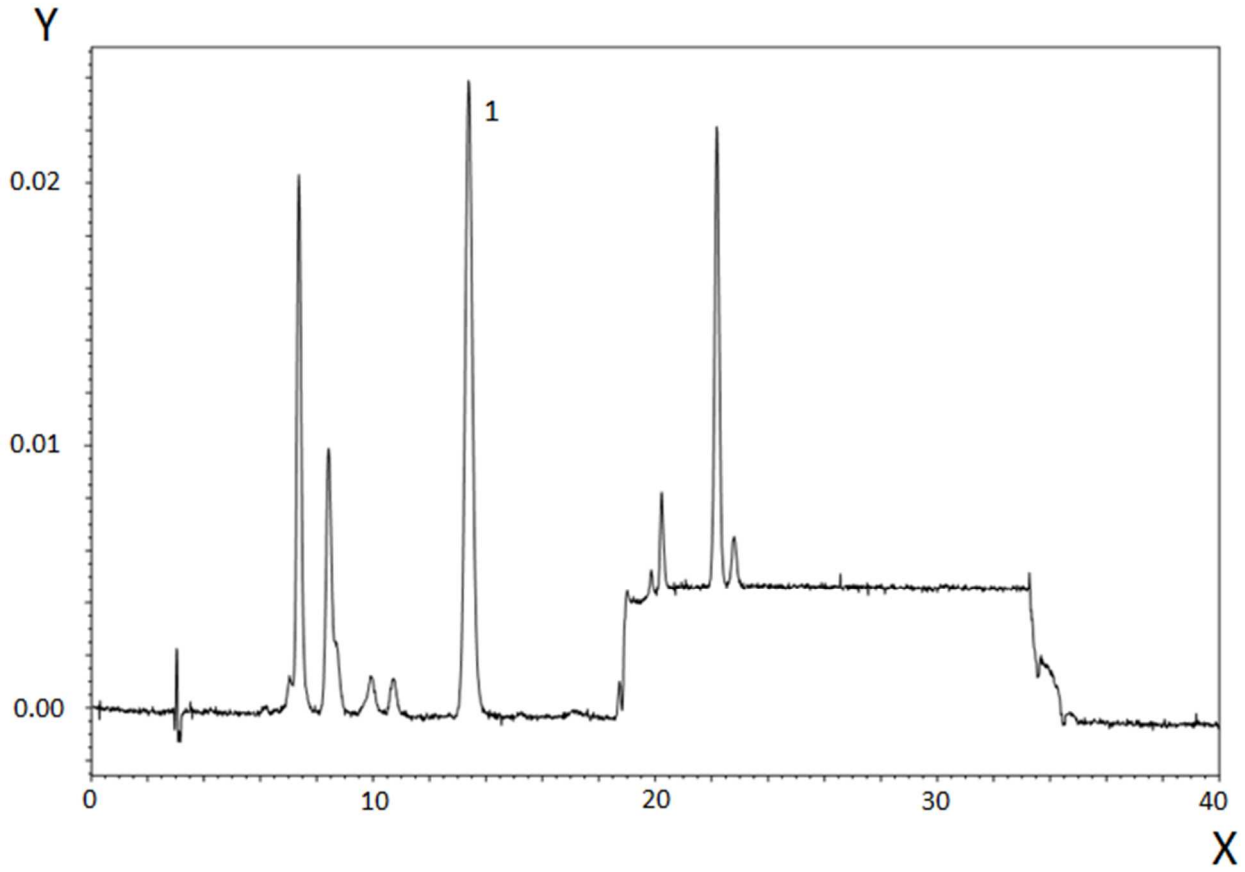
1) The fluctuation between 18 min and 33 min coused from switching of flow lines. There are cases that some baselines are high and others are low.

2) YMC® Carotenoid is an example of a suitable column available commercially. This information is given for the convenience of users of this standard and does not constitute an endorsement of this product.




**Key**

X Retention time, min
Y Response, AU

1 Lutein

**Figure B.2 − 'Spinach' extract**

**HPLC Condition**

HPLC operating conditions in accordance with **7.4.1** and the following.

a) Chromatographic column : YMC® Carotenoid

b) Binary gradient conditions: **Table 1**

# Annex C
# (informative)
# The procedure of purifying lutein

## C.1 General

This annex specifies a procedure of purifying lutein by silica gel column chromatography that is used as standard stock solutions in the interlaboratory test described in annex A.

## C.2 Reagents

**a)** **Packing materials,** with a particle size of 150 μm to 425 μm, for column chromatography.

**b)** **Lutein,** specified in **4.2**.

**c)** **Acetone**, according to **JIS K 8034**.

**d)** **Hexane,** specified in **4.7**.

**e)** **Sodium sulfate,** according to **JIS K 8987**.

**f)** **Ethyl acetate,** specified in **4.8**.

**g)** **Nitrogen,** specified in **4.13**.

**h)** **Eluent A**, specified in **4.18**.

**i)** **Eluent B**, mix 7 parts per volume of hexane (**4.7**) with 3 part per volume of ethyl acetate (**4.8**).

**j)** **Eluent C**, mix 6 parts per volume of hexane (**4.7**) with 4 part per volume of ethyl acetate (**4.8**).

## C.3 Apparatus

**a)** **Chromatographic tubes,** cylindrical glass tube, of 15 mm to 20 mm diameter and more than 30 cm length, with a stopcock.

**b)** **Wools,** absorbent cotton or glass wool, plug in the bottom of chromatography tubes (**C.3 a)**) and capable of preventing outflow of packing materials (**C.2 a)**).

**c)** **Round-bottomed flasks,** specified in **5.10**.

**d)** **Vacuum rotating evaporator,** specified in **5.11**.

## C.4 Procedure

**a)** Mix about 10 g of packing materials (**C.2 a)**) and a small amount of hexane. Place wool (**C.3 b)**) in the bottom of a chromatographic tube (**C.3 a)**) and then poured the mixture into it.

**b)** Prepare a lutein (**4.2**) solution in acetone (**4.11**). Transfer this solution into the chromatographic tube (**C.4 a)**). Lutein solutions can be mixed with a small amount of packing materials, then evaporate its solvent before transferred into the chromatographic tube (**C.4 a)**).

**c)** Place about 3 g of sodium sulfate (**C.2 e)**) above the adsorbent layer in the chromatographic tube (**C.4 b)**).

**d)** Add 100 ml of eluent A (**C.2 h)**), 100 ml of eluent B (**C.2 i)**), 100 ml of eluent C (**C.2 j)**) and 100 ml of ethyl acetate (**4.8**) in this order. Collect orange-coloured eluent in the round-bottomed flask (**5.10**).

**NOTE** The colour of eluent changes as colourless, yellow, orange and yellow in this order.

**e)** Evaporate almost solvent in a round-bottomed flask (**C.4 d)**) at less than 40 °C by vacuum rotating evaporator (**5.11**). Then evaporate the solvent just under a gentle stream of nitrogen.

# Bibliography

[1] **ISO 5725-1:1994** Accuracy (trueness and precision) of measurement methods and results — Part 1:General principle and definitions

**NOTE** The expression of the repeatability limit and reproducibility limit referred to section 7.1.5.

[2] **ISO 5725-6:1994** Accuracy (trueness and precision) of measurement methods and results — Part 6:Use in practice of accuracy value

**NOTE** The calculation of the repeatability limit and reproducibility limit referred to section 4 “Determination of limits”.

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

[4] Thompson, M., et al., The International Harmonized Protocol for the Proficiency Testing of Analytical Chemistry Laboratories. *Pure Appl.Chem*. 78(1), 145-196 (2006)

**NOTE** The method of homogeneity referred to section 3.11 “Testing for sufficient homogeneity and stability”.

[5] Britton, G., Liaaen-Jensen, S., Pfander, H. ed.,*Carotenoids handbook*, Birkhauser Verlag, Basel/Boston/Berlin, 2004

**NOTE** The absorption coefficient of lutein referred to “MAIN LIST 133(Lutein) Spectroscopic data”.