Tentative Translation

JAS 0003

JAPANESE AGRICULTURAL STANDARD

Determination of the β-cryptoxanthin in Satsuma Mandarin — High-performance liquid chromatographic method

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Incorporated Administrative Agency
Food and Agricultural Materials Inspection Center

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Determination of the β-cryptoxanthin in Satsuma Mandarin — High-performance liquid chromatographic method

Warning — The user of 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 β-cryptoxanthin (BCR) in the edible part of Satsuma Mandarin (*Citrus unshiu* Marc.) (fresh fruits).

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
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 8101 Ethanol (99.5)
JIS K 8150 Sodium chloride
JIS K 8361 Ethyl acetate (Reagent)
JIS K 8574 Potassium hydroxide
JIS K 8593 Petroleum ether (Reagent)
JIS K 8780 Pyrogallol
JIS K 8839 2-Propanol (Reagent)
JIS K 8848 Hexane (Reagent)
JIS K 8987 Sodium sulfate

3 Principle

The BCR content from a test portion of ground sample is extracted with ethanol. The extract is saponified with potassium hydroxide. The unsaponifiable matter is extracted by hexane and ethyl acetate mixture. The BCR in the extract are 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 BCR, of minimum mass fraction, $\varphi(C_{40}H_{56}O) \geq 99$ % (HPLC).

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

4.4 Pyrogallol, of minimum mass fraction, $\varphi(C_6H_6O_3) \geq 99,5$ %, according to **JIS K 8780**.

4.5 Sodium sulfate, of minimum mass fraction, $\varphi(Na_2SO_4) \geq 99,0$ %, according to **JIS K 8987**.

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

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

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

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

4.10 2-Propanol, of minimum mass fraction, $\varphi((CH_3)_2CHOH) \geq 99,7$ %, according to **JIS K8839.**

4.11 Methanol, HPLC grade.

4.12 Chloroform, HPLC grade.

4.13 Ascorbyl palmitate, of minimum mass fraction, 97,0%.

4.14 Petroleum ether, special grade stipulated in **JIS K 8593** or those of quality equivalent or superior.

4.15 Nitrogen, of volume fraction, $(N_2) \geq 99,5\%$.

4.16 β– carotene, of minimum mass fraction, 90%.

4.17 30 g/l pyrogallol, ethanolic standard volumetric solutions. Dissolve 30 g of pyrogallol (**4.4**) per 1 l of ethanol (**4.3**). Do not use pyrogallol solution changed to brown color.

4.18 Potassium hydroxide (water) solution, corresponding to approximately 60 %.

Dissolve 60 g of potassium hydroxide (**4.6**) 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.

4.19 Sodium chloride (water) solution, corresponding to approximately 1 %.

Dissolve 10 g of sodium chloride (**4.7**) per 1 l of water (**4.1**).

4.20 Hexane/ethyl acetate mixture,

Mix 9 parts per volume of hexane (**4.8**) with 1 part per volume of ethyl acetate (**4.9**).

4.21 HPLC mobile phase,

Mix 24 parts per volume of methanol (**4.11**) with 1 part per volume of chloroform (**4.12**). Dissolve 0,05 g of ascorbyl palmitate **(4.13)** per 1 l of that solution.

NOTE In the interlaboratory tests described in **Annex A**, after dissolve 0,05 g of ascorbyl palmitate in 960 ml of methanol, 40 ml of chloroform was added.

4.22 BCR stock standard solution,

Prepare a solution containing BCR (**4.2**) in petroleum ether (**4.14**) at a concentration of 10 μg/ml (for example, 100 ml of petroleum ether containing 1 mg of BCR). Transfer this standard into a labelled bottle with screw cap and store.

NOTE In the interlaboratory tests described in **Annex A**, BCR stock standard solution was stored at –30 °C to –20 °C. It has been confirmed that the standard stock solution when stored frozen at –30 °C to –20 °C will remain stable for at least half a year.

Remove from freezer before use, allow it to attain to room temperature and mix. The small amount of particulate material that may settle during storage is removed using a membrane filter (**5.10**).

4.23 Standard solutions

4.23.1 General

Prepare BCR standard solutions of 4 or more stepwise concentrations.

A standard solution for absorbance measurement (**4.23.2**) and a series of the standard solutions (**4.23.3**), should be prepared from a single bottle of the BCR stock standard solution (**4.22**). Every time BCR 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 resave.

4.23.2 Standard solution for absorbance measurement

Dilute the BCR stock standard solution (**4.22**) 5-fold with petroleum ether using the single volume pipette (**5.5**) and volumetric flask (**5.6**).

NOTE In the interlaboratory tests described in **Annex A**, 2 ml of BCR stock standard solution was transferred into a 10 ml volumetric flask.

Set up and operate the spectrometer (**5.12**) in accordance with the manufacturer's instructions. Measure the concentration of standard solution for absorbance measurement by using a spectrometer at 452 nm with a petroleum ether as reference. The BCR concentration of the stock standard solution in μg/ml, ρ_1, is given by the formula:

$$\rho_1 = \frac{A \times V_2 \times 10\ 000}{\varepsilon V_1}$$

where

A is the absorbance of standard solution for absorbance measurement determined at 452 nm (petroleum ether, 1 cm cell);

ε is the absorption coefficient of BCR in concentration 1 % and optical pathlength 1 cm, 2 386[6] [7];

V_1 is used single volume pipette's capacity, in ml, in the interlaboratory tests, that is, 2;

V_2 is used one-mark volumetric flask's capacity, in ml, in the interlaboratory tests, that is, 10.

After calculation, proceed immediately in accordance with **4.23.3**.

4.23.3 A series of standard solutions

Using each single volume pipette (**5.5**), transfer each quantity of BCR stock standard solution (**4.22**) into round-bottomed flasks (**5.8**). Evaporate the solutions just to dryness under a stream of nitrogen (**4.15**) gently. Dissolve the contents in each of the round-bottomed flasks completely by ethanol (**4.3**) (For example, using ultrasonic during about 10 sec). Transfer the solution completely to each volumetric flasks (**5.6**). Add to the mark with ethanol and mix. Filter through a membrane filter (**5.10**) and transfer filtrate into vials (**5.11**). The BCR concentration of the dilute standard solution, in μg/ml, ρ_i, is given by the formula:

$$\rho_i = \frac{\rho_1 \times V_3}{V_4}$$

where

ρ_1 is the concentration of BCR stock standard solution (**4.22**) (see **4.23.2**), in μg/ml;

V_3 is used single volume pipette's capacity, in ml:

V_4 is used one-mark volumetric flask's capacity, in ml.

NOTE 1 In the interlaboratory tests described in **Annex A**, each standard solutions were prepared as **Table 1**.

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

Standard solutions	Single volume pipette's capacity (ml)	One-mark volumetric flask's capacity (ml)	BCR standard solutions concentration for HPLC (equivalent to μg/ml)
A	1	5	2,0
B	1	10	1,0
C	0,5	10	0,50
D	0,5	20	0,25
NOTE The values given are for guidance only.			

Perform HPLC analysis (**7.5.2**) on the day of preparation, or store them frozen.

NOTE 2 It has been confirmed that the standard solutions will remain stable for at least a week when stored frozen at -30 °C to -20 °C.

Remove each frozen stored standard solution from freezer before HPLC analysis (**7.5.2**), allow it to attain to room temperature and mix it thoroughly. Dissolve the insoluble matter in the solution by ultrasonic as necessary, and filter through a membrane filter.

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, round-bottomed, of 50 ml capacity, stoppered, keeping the space for enough mix and centrifugation at 400×g is possible. It should be clear glass. Use a lid with packing made from PTFE or other materials resistant to organic solvents and strong basic solutions.

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 Single volume pipettes, to cover the volume range for standard and sample saponification, of **ISO648**, class A.

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

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

5.8 Round-bottomed flask, of 100 ml capacity, with ground neck, usable evaporation.

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

5.10 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.

5.11 Vials, suitable for HPLC to be used. 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 made from PTFE.

5.12 Spectrometer, capable measuring wavelengths 452 nm, holding cells (**5.13**).

5.13 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.

5.14 HPLC apparatus

5.14.1 High-performance liquid chromatograph (HPLC), equipped to perform with a degassing unit, a thermostatically controlled column compartment, an auto sampler, an ultraviolet detector set at 455 nm, and data collection/integration system, according to **JIS K 0124**.

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

— length: 150 mm

— internal diameter: 4,6 mm

— spherical particle size: 3 μm to 5 μm

— β-carotene shall be eluted within 20 min. Confirm that elution time of β-carotene and β-carotene peak do not overlap with BCR peak according to **7.5**. When a guard column is used, select the one matching to the C18 (ODS) column.

6 Preparation of test samples

After removing only the outer peel of the sample, it is pulverized using a homogenizer or the like.

Proceed immediately in accordance with **7.1**, or store frozen the test samples.

If test samples are stored frozen, transfer them into the glass sealed containers soon after pulverized. Remove the test samples from the freezer before use, allow them to room temperature and mix well.

NOTE 1 It has been confirmed that the samples of fresh fruit will remain stable for 2 weeks when stored refrigerate[3].

NOTE 2 It has been confirmed that the test samples will remain stable for at least 2 months when store frozen at –20 °C or lower.

7 Procedure

7.1 Extraction

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

Add 15 ml of pyrogallol (**4.17**) and 10 g of sodium sulfate (**4.5**) to the tube.

7.1.2 Mix hard the tube 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 50 ml volumetric flask（**5.6**）.

7.1.3 Add 15 ml of pyrogallol to the liquid left in the tube. Repeat steps **7.1.2**. Transfer the supernatant into the same one-mark volumetric flask which the **7.1.2** supernatant was transferred.

7.1.4 Repeat extraction steps **7.1.3**.

7.1.5 Make up to the mark with pyrogallol and mix.

7.2 Saponification

Using a single volume pipette (**5.5**), transfer 10 ml of extracts (**7.1.5**) into a tube (**5.2**). Add 1 ml of potassium hydroxide (water) solution (**4.18**) to the tube, mix gently. Heat the tube for 30 min on a constant temperature water bath (**5.7**) set at 70 °C while mixing every 5 min. Cool the tube to room temperature on a water tank containing tap water.

7.3 Extraction of unsaponifiable matter

7.3.1 Add 20 ml of sodium chloride (water) solution (**4.19**), 5 ml of 2-propanol (**4.10**) and 12 ml of hexane / ethyl acetate mixture (**4.20**) to the tube (**7.2**) and mix.

7.3.2 Mix hard the tube 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 (**5.8)**.

7.3.3 Add 12 ml of hexane/ethyl acetate mixture to the liquid left in the tube. Repeat steps **7.3.2**. Transfer the supernatant into the same round-bottomed flask which the **7.3.2** supernatant was transferred.

7.3.4 Repeat steps **7.3.3**.

7.3.5 Evaporate almost the solvent in a round-bottomed flask (**7.3.4)** at less than 40 °C by rotary evaporator (**5.9**). Then evaporate the solvent just to dryness under a stream of nitrogen (**4.15**) gently.

7.4 Dissolution

Dissolve the contents of the round-bottom flask (**7.3.5**) completely (for example, using ultrasonic for 10 sec) by ethanol (**4.3**). Transfer the solution completely to a one-mark volumetric flask (**5.6**).

NOTE 1 5 ml of one-mark volumetric flasks were used in the interlaboratory tests described in **Annex A**.

Add to the mark with ethanol and mix. Filter through a membrane filter and transfer filtrate into the vials (**5.11**).

Perform HPLC measurement (**7.5.2**) on the day of preparation or store the sample extract frozen.

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

Remove the stored sample extract from the freezer allow it to attain to room temperature on the day of measurement. Mix using a vortex mixer or the like to sufficiently dissolve the insoluble matter (for example, using ultrasonic another for 10 sec). Filter through a membrane filter.

7.5 Determination

7.5.1 HPLC operating conditions

Set up the chromatograph (**5.14**) in accordance with the manufacturer's instructions and adjust it as follows. The automatic sample injector with cooling function should set at 20 °C.

a) Flow rate of the mobile phase (**4.21**): 1,5 ml/min

b) Temperature of the column (**5.14.2**) set at 40 °C

c) UV detector set at 455 nm.

d) Volume injected: 20 µl

e) Time: 25 min

If the next measurement is not disturbed by a peak of β-carotene, the time may be cut.

7.5.2 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 BCR by a blank run under the specified condition (**7.5.1**). Then inject a series of standard solutions (**4.23.3**) onto the column, followed by an equal volume of the sample extract solutions (**7.4**).

7.5.3 Identification

Identify the individual BCR by comparing retention times from sample chromatograms with those obtained from the standard solutions under the same chromatographic conditions (**7.5.1**).

NOTE Typical HPLC chromatogram of a Satsuma Mandarin 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 a impurities, take appropriate measures according to the perpendicular or tangent method prescribed by **JIS K 0124**. For the shoulder peaks derived from isomers, their peak areas are summed up as the peak area of BCR.

8.2 Quantitation

Calculate the concentration (μg/ml) of BCR in each of a series of standard solutions (**4.23.3**). Construct linear calibration graphs for each standard from the BCR concentrations (μg/ml) against the peak areas obtained from the data collection/integration system (**5.14.1**). The correlation coefficient of the linear calibration is required to be more than 0,995.

Calculate the concentration (μg/ml) of BCR in the individual sample solution by using the linear calibration. The BCR content, w_i, expressed as a percentage by mass on Satsuma Mandarin sample, is given by the formula:

$$w_i = \frac{C \times V_5 \times d_1}{W \times d_2}$$

where

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

V_5 is the constant volume (ml) at the time of dissolution of recovered unsaponifiable matter (**7.4**), in the interlaboratory tests, that is, 5;

d_1 is the constant volume (ml) at the time of extraction (**7.1**), typically 50;

d_2 is the saponification (**7.2**) fraction volume (ml), typically 10;

W is the mass, in grams, of the sample test portion.

8.3 Expression of results

Express the results to two significant figures (e.g., 11 mg/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 (4,7 mg/kg to 23 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[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 expected 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 date 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**[4]. Some samples from which the outer peel of commercially available Satsuma Mandarin was removed, 150 to 200 grams, were added pyrogallol with 10 % of sample mass as an antioxidant, and were grinded at 12 000 r/min for 10 min by grinding device.

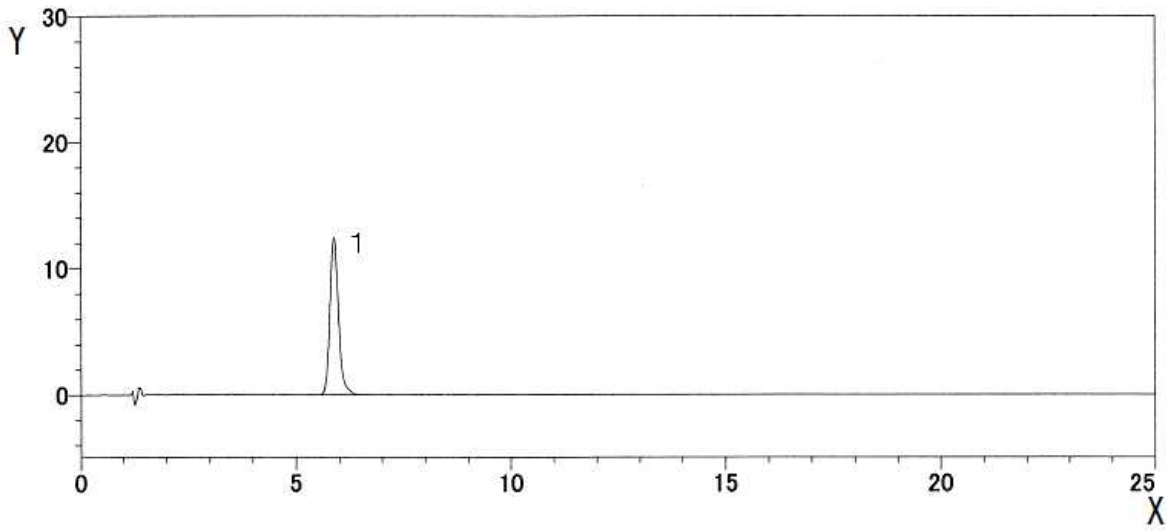
NOTE Since carotenoids may be decomposed by light, oxygen, enzymes contained in samples, antioxidants are added.

Homogeneity [5] was confirmed and used as a test sample. 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	Sample 2	Sample 3	Sample 4	Sample 5
Number of participating laboratories	11	11	11	11	11
Number of accepted test results	10	9	9	9	10
Mean BCR content, mg/kg (mass fraction),	4,73	6,75	10,2	13,7	23,4
Repeatability standard deviation s_r mg/kg	0,12	0,13	0,32	0,57	0,50
Repeatability relative standard deviation, %	2,6	2,0	3,1	4,2	2,1
Repeatability limit r (r = 2,8 s_r) mg/kg	0,34	0,36	0,90	1,6	1,4
Reproducibility standard deviation s_R mg/kg	0,67	0,61	1,0	1,3	2,6
Reproducibility relative standard deviation, %	14	9,0	9,9	9,6	11
Reproducibility limit R (R = 2,8 s_R) mg/kg	1,9	1,7	2,8	3,6	7,3

Annex B
(informative)
Typical HPLC chromatograms



Key

X Retention time, min

Y Response, mV

1 BCR

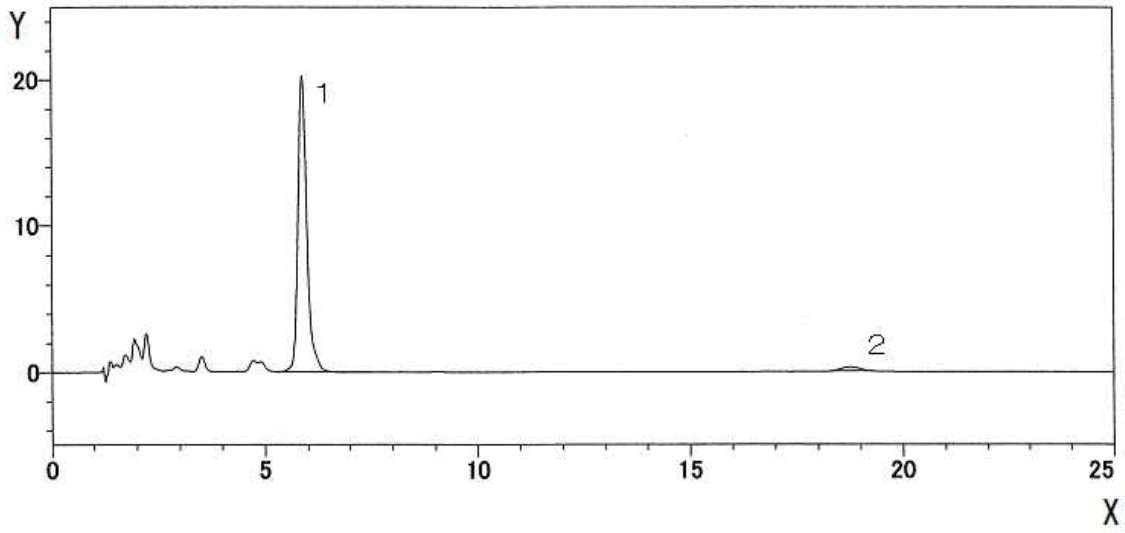
FigureB.1 − BCR standard B

HPLC operating condition

HPLC operating conditions in accordance with **7.5.1** and the following.

a) Chromatographic column : Inertsil® ODS-3[1)]

1) Inertsil® 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, mV

1 BCR

2 β-carotene

Figure B.2 − Satsuma Mandarin extract

HPLC operating condition

HPLC operating conditions in accordance with **7.5.1** and the following.

a) Chromatographic column : Inertsil® ODS-3[2)]

2) Inertsil® 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.

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