Tentative Translation

JAS 0023

JAPANESE AGRICULTURAL STANDARD

Testing method of K-value as a freshness index for fish
－High performance liquid chromatographic method

Date of Establishment: 2022 – 3 – 31

Ministry of Agriculture, Forestry and Fisheries

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Food and Agricultural Materials Inspection Center, Incorporated Administrative Agency

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Foreword

This Japanese Agricultural Standard has been established by the Minister of Agriculture, Forestry and Fisheries through deliberations at the Council for the Japanese Agricultural Standards as the result of proposal for establishment of Japanese Agricultural Standard submitted by the Hakodate Regional Industry Promotion Organization, Public Interest Incorporated Foundation with the original bill being attached, based on the provision of Article 4, paragraph (1) of the Act on Japanese Agricultural Standards.

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JAPANESE AGRICULTURAL STANDARD JAS
(Tentative Translation) 0023 : 2022

Testing method of K-value as a freshness index for fish

—High performance liquid chromatographic method

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

1 Scope

This document specifies a test method for calculating the K-value, which is a freshness index, from the content of ATP-related compounds in fresh fish (limited to Osteichthyes, and excluding thawed fish) measured by high performance liquid chromatography.

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. The latest edition of the referenced document (including any amendments) applies.

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 8223, *Perchloric acid (Reagent)*

JIS K 9005, *Phosphoric acid*

JIS K 9009, *Sodium dihydrogenphosphate dihydrate (Reagent)*

JIS K 9020, *Disodium hydrogenphosphate (Reagent)*

JIS Z 8802, *Methods for determination of pH of aqueous solutions*

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*

3 Terms and definitions

For the purposes of this document, the following terms and definitions apply.

3.1
ATP-related compound
adenosine 5'-triphosphate (ATP), adenosine 5'-diphosphate (ADP), adenosine 5'-monophosphate (AMP), inosine 5'-monophosphate (IMP), inosine (HxR) and hypoxanthine (Hx)

3.2

K-value
ratio of the sum of the contents of HxR and Hx to the sum of the contents of the ATP-related compounds in test samples expressed as a percentage [1]

Note 1 to entry: See 9.2.3, Formula [3]

4 Principle

The endogenous enzymes that decompose ATP-related compounds are inactivated and ATP-related compounds are extracted with dilute perchloric acid. The contents of the ATP-related compounds in the sample solution are measured using a high performance liquid chromatograph (hereinafter referred to as HPLC) equipped with an ultraviolet-visible absorbance detector. The K-value is calculated from those contents [1], [2].

5 Reagents

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

WARNING — It is the responsibility of users of this document to comply with legal regulations regarding the use of reagents.

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

5.2 Perchloric acid, of mass fraction 60,0 % to 62,0 %, specified in JIS K 8223.

5.3 Phosphoric acid, of minimum mass fraction, 85,0 %, specified in JIS K 9005.

5.4 Sodium dihydrogenphosphate dihydrate, of mass fraction 99,0 % to 102,0 %, specified in JIS K 9009.

5.5 Disodium hydrogenphosphate, of minimum mass fraction, 99,0 %, specified in JIS K 9020.

5.6 Standards of ATP-related compounds, are given in Table 1. Those purity shall be confirmed by separating impurities (including other ATP-related compounds) with HPLC. The purity may be confirmed by the supplier of the standards or the user of this document.

Table 1— Standards of ATP-related compounds

ATP-related compound	Name of standards	CAS number	purity
ATP	Adenosine 5'-triphosphate disodium salt hydrate	34369-07-8	98 % or more
ADP	Adenosine 5'-diphosphate monopotassium salt dihydrate	72696-48-1	95 % or more
AMP	Adenosine 5'-monophosphate sodium salt hydrate	149022-20-8	98 % or more
IMP	Inosine 5'-monophosphate disodium salt hydrate	352195-40-5	98 % or more
HxR	Inosine	58-63-9	98 % or more
Hx	Hypoxanthine	68-94-0	98 % or more

NOTE In the interlaboratory tests described in Annex A, adenosine 5'-triphosphate disodium salt hydrate of formula weight 551,1(anhydrous basis), adenosine 5'-diphosphate monopotassium salt dihydrate of formula weight 501,3, adenosine 5'-monophosphate sodium salt hydrate of formula weight 347,2(anhydrous free acid basis), inosine 5'-monophosphate disodium salt hydrate of formula weight 392,2(anhydrous basis), inosine of formula weight 268,2 and hypoxanthine of formula weight 136,1 were used.

5.7 Dilute perchloric acid, prepared by adding 40,0 g of perchloric acid to 440 mL of water.

5.8 Sodium hydroxide (water) solution, prepared to a concentration of about 1 mol/L to 5 mol/L in water. A commercially available sodium hydroxide solution of the same concentration may be used.

5.9 Dilute hydrochloric acid, prepared to a concentration of about 1 mol/L in water. A commercially available dilute hydrochloric acid of the same concentration may be used.

5.10 Phosphate buffer solutions

5.10.1 0,25 mol/L Phosphate buffer solution (pH7,0)

Prepare 0,25 mol/L disodium hydrogenphosphate solution by diluting disodium hydrogenphosphate in water. Prepare 0,25 mol/L sodium dihydrogenphosphate solution by diluting sodium dihydrogenphosphate dihydrate in water. Add 0,25 mol/L sodium dihydrogenphosphate solution to 0,25 mol/L disodium hydrogenphosphate solution until pH of the mixture reaches 7,0.

NOTE This buffer is used in the case using a silica gel-based column specified in 6.1.2 a).

5.10.2 0,05 mol/L Phosphate buffer solution (pH7,0)

Prepare 0,05 mol/L disodium hydrogenphosphate solution by diluting disodium hydrogenphosphate in water. Prepare 0,05 mol/L sodium dihydrogenphosphate solution by diluting sodium dihydrogenphosphate dihydrate in water. Add 0,05 mol/L sodium dihydrogenphosphate solution to 0,05 mol/L disodium hydrogenphosphate solution until pH of the mixture reaches 7,0.

5.10.3 1 mol/L Phosphate buffer solution (pH2,9)

Prepare 1 mol/L sodium dihydrogenphosphate solution by diluting sodium dihydrogenphosphate dihydrate in water. Prepare 1 mol/L phosphoric acid solution by diluting phosphoric acid in water. Add 1 mol/L phosphoric acid solution to 1 mol/L sodium dihydrogenphosphate solution until pH of the mixture reaches 2,9.

NOTE This buffer is used in the case using a polymer-based column specified in 6.1.2b).

5.10.4 0.2 mol/L Phosphate buffer solution (pH2,9)

Prepare 0,2 mol/L sodium dihydrogenphosphate solution by diluting sodium dihydrogenphosphate dihydrate in water. Prepare 0,2 mol/L phosphoric acid solution by diluting phosphoric acid in water. Add 0,2 mol/L phosphoric acid solution to 0,2 mol/L sodium dihydrogenphosphate solution until pH of the mixture reaches 2,9.

NOTE This buffer is used in the case using a polymer-based column specified in 6.1.2 b).

5.11 Stock standard solutions of ATP-related compounds

Prepare ATP stock standard solution, ADP stock standard solution, AMP stock standard solution, IMP stock standard solution, HxR stock standard solution and Hx stock standard solution respectively, by adding each standard of ATP-related compounds specified in 5.6 to water to a concentration of about 5 mmol/L. Among those, Hx stock standard solution may be prepared by adding hypoxanthine to water heated about 70 °C and allowing it to cool down.

NOTE It has been confirmed that hypoxanthine is difficult to dissolve in cool water at the concentration given in this subclause.

5.12 Standard solutions

5.12.1 Standard solutions for absorbance measurement

Prepare ATP standard solution for absorbance measurement, ADP standard solution for absorbance measurement, AMP standard solution for absorbance measurement, IMP standard solution for absorbance measurement, HxR standard solution for absorbance measurement and Hx standard solution for absorbance measurement respectively, by diluting each stock standard solution of the ATP-related compounds 100-fold with 0,05 mol/L phosphate buffer solution (pH7,0) (see 5.10.2), using single volume pipettes or piston pipettes, and one-mark volumetric flasks.

NOTE In the interlaboratory tests described in Annex A, 1 mL of the stock standard solutions were poured into 100 mL one-mark volumetric flasks with single volume pipettes, and 0,05 mol/L phosphate buffer solution (pH7,0) was added to the mark.

Set up and operate the spectrophotometer (see 6.2) in accordance with the manufacturer's instructions. Measure the absorbance of standard solutions for absorbance measurement at wavelengths given in Table 2 with 0,05 mol/L phosphate buffer solution (pH7,0) as reference. The concentration of the ATP-related compound X in each stock standard solution, $c_{X,\,std,}$ is given by the following formula:

$$c_{X,std} = \frac{A(\lambda_X)}{\varepsilon_X \times l} \times 10^6 \times \frac{V_1}{V_2} \quad (1)$$

where

$c_{X,\,std}$ is the concentration of the ATP-related compound X in the stock standard solution (μmol/L);

$A(\lambda_X)$ is the absorbance of the ATP-related compound X standard solution for absorbance measurement determined at measurement wavelengths λ_X given in Table 2;

ε_X is the molar absorption coefficient of the ATP-related compound X (mol^{-1} L cm^{-1}) (see Table 2);

l is the optical path length (cm) of the absorption cell (see 6.3);

V_1 is used volumetric flask's capacity (mL) ;

V_2 is the volume (mL) of the stock standard solution used.

Table 2—Measurement wavelengths and molar absorption coefficients of ATP-related compounds [3]

ATP-related compound	Measurement wavelength (nm)	Molar absorption coefficient (mol^{-1} L cm^{-1})
ATP	259	15 400
ADP	259	15 400
AMP	259	15 400
IMP	249	12 700
HxR	249	12 300
Hx	250	10 700
NOTE Results of studies on the molar absorption coefficient of HxR at pH7,0 are shown in Annex B.		

5.12.2 A series of standard working solutions

Using single volume pipettes or piston pipettes, pour ATP stock standard solution, ADP stock standard solution, AMP stock standard solution, IMP stock standard solution, HxR stock standard solution and Hx stock standard solution into a one-mark volumetric flask, and water is added to the mark. It is used as the mixed stock standard working solution. Depending on the type of column specified in 6.1.2, prepare a series

of standard working solutions with a concentration of 4 or more stepwise from the mixed stock working standard solution by one of the following steps.

a) For using a silica gel-based column: Dilute the mixed stock standard working solution with water using a single volume pipette or a piston pipette. Mix 4 parts per volume of this diluted solution with 1 part per volume of 0,25 mol/L phosphate buffer solution (pH7,0) (see 5.10.1) to prepare the standard working solutions.

b) For using a polymer-based column: Dilute the mixed stock standard working solution with water using a single volume pipette or a piston pipette. Mix 4 parts per volume of this diluted solution with 1 part per volume of 1 mol/L phosphate buffer solution (pH2,9) (see 5.10.3) to prepare the standard working solutions.

NOTE 1 In the interlaboratory tests described in Annex A, 1,25 mL of each standard working solution was prepared. Their concentrations were about 0,8 µmol/L, about 3,2 µmol/L, about 8 µmol/L, about 32 µmol/L, about 160 µmol/L, about 320 µmol/L and about 480 µmol/L.

NOTE 2 It has been confirmed that the standard working solutions will remain stable for at least 7 days when kept at 10 °C.

6 Apparatus

Usual laboratory apparatus and, in particular, the following.

6.1 HPLC system

6.1.1 High performance liquid chromatograph, equipped with a liquid feeding pump, a thermostatically controlled column compartment (column oven), an ultraviolet-visible detector set at 260 nm, and data collection/integration system specified in JIS K 0124. It should have a degas chamber at a mobile phase feeding unit, and a cooling system at a sample injector.

6.1.2 Column, silica gel-based or polymer-based and with characteristics that the peaks of the ATP-related compounds are detected within 60 minutes and that the resolution specified in JIS K 0124 is 1,5 or higher when the minimum concentration of a series of standard working solutions is measured according to 8.6. When using a guard column, select the one with the same packing material as the column used for the measurement. The specifications given in a) and b) are available.

a) Silica gel-based column:

- packing material: adamantyl group introduced silica gel;
- material of chromatographic tube: stainless steel;
- length: 250 mm;
- internal diameter: 4,6 mm;
- spherical particle size: 5 µm.

NOTE Some of octadecyl group introduced silica gel columns have been confirmed that the peaks of HxR or Hx overlap with the peaks of impurities when the sample solution is measured, and the K-value is calculated higher than actual value.

b) Polymer-based column:

- packing material: polyvinyl alcohol-based gel with a pore size of 40 nm;
- material of chromatographic tube: stainless steel;
- length: 300 mm;
- internal diameter: 7,5 mm;

— spherical particle size: 6 μm.

6.2 Spectrophotometer, capable of measuring at wavelengths of 249 nm, 250 nm and 259 nm, capable of holding cells of optical path length 1 cm, specified in JIS K 0115.

6.3 Cells, quartz glass, of optical path length 1 cm.

6.4 One-mark volumetric flasks, with the precision equivalent or higher than class A of ISO 1042, covering the volume range of solution preparation.

6.5 Single volume pipettes, with the precision equivalent or higher than class A of ISO 648.

6.6 Piston pipettes, suitable for the dilution of standard solutions, etc., of ISO 8655-2.

6.7 Extraction container, capable of being used in the extraction (see 8.2) process, and resistant to solutions to be used.

6.8 Membrane filters, made of hydrophilic polytetrafluoroethylene (PTFE) suitable for acidic solutions, pore size of 0,45 μm or less. The filter and housing shall be unitary, and the housing material shall be resistant to acidic solutions, Capable of being used with a syringe attached.

6.9 Electronic analytical balance, capable of weighing to an accuracy of ±10 mg, and capable of weighing more than 200 g.

6.10 Homogenizer, capable of stirring test samples and reagents, and suspending test samples in reagents, in the extraction (see 8.2) process.

6.11 pH meter, specified in JIS Z 8802.

6.12 pH test paper, capable of identifying pH2,5 to pH3,5.

7 Preparation of test samples

In the case that heads, bones, viscera, epidermis, fins, etc. are found, remove them and obtain the lateral muscle. Remove dark meat, collect ordinary meat and homogenize it with a food processor, etc. Use it as a test sample. Immediately perform the operation in 8.1. For frozen samples, perform the operation in this clause immediately after thawing or partial thawing.

8 Procedure

8.1 General

In order to avoid decomposition of ATP-related compounds, cool samples and extracts with ice and keep them at low temperature until the operation in 8.4 is performed, except during the experimental operation.

8.2 Extraction

Weigh, to the nearest 10 mg, approximately 2,0 g of the test sample (see Clause 7) into an extraction container, add ice-cooled dilute perchloric acid. Using homogenizer, stir and suspend the sample in the dilute perchloric acid. In the case the suspension adheres to the homogenizer, rinse it off with the ice-cooled dilute perchloric acid and mix both well to make a suspended extract. The total amount of the dilute perchloric acid to be used is about 30 mL.

NOTE In the interlaboratory tests described in Annex A, 20 mL of ice-cooled dilute perchloric acid was added to the sample in a 50 mL polypropylene tube and was stirred with a rotor-stator type homogenizer at a rotation speed of 10 000 min^{-1} for 30 seconds. Subsequently, the homogenizer shaft was washed with 10 mL of ice-cooled dilute perchloric acid, and both were mixed to obtain a suspended extract.

8.3 pH adjustment [4]

8.3.1 Add sodium hydroxide solution to the suspended extract (see 8.2) and stir it to adjust the pH to 2,5

to 3,5. Check the pH with a pH meter or pH test paper. In the case the pH exceeds 3.5, add dilute hydrochloric acid to adjust the pH.

NOTE In the interlaboratory tests described in Annex A, 2,7 mL of 5 mol/L sodium hydroxide solution was added to a suspended extract and stirred with a glass rod. A small portion of this solution was collected and the pH was checked with pH test paper. In the case the pH was 2,5 or less, 100 µL of 1 mol/L sodium hydroxide solution was added, stirred, and the pH was checked again. In the case the pH exceeds 3,5, 100 µL of dilute hydrochloric acid was added, and the pH was checked. This process was repeated until the pH reached 2,5 to 3,5.

8.3.2 Rinse the residue with water, transfer the entire pH-confirmed suspension extract (see 8.3.1) to a one-mark volumetric flask. Add water to the capacity mark with water and shake to mix it.

NOTE In the interlaboratory tests described in Annex A, 50 mL one-mark volumetric flasks were used.

8.3.3 Collect all or a portion of the content of the one-mark volumetric flask (see 8.3.2) and cool the collection with ice for at least 30 minutes. Use this as a pH adjusted extract.

8.4 Removal of protein

Filter a portion of the supernatant from the pH adjusted extract (see 8.3.3) through a membrane filter to obtain a filtrate.

8.5 Preparation of sample solution

Mix 4 parts per volume of the filtrate (see 8.4) with 1 part per volume of the phosphate buffer solution with single volume pipettes or piston pipettes. Use this as a sample solution. Use one of the following as the phosphate buffer solution:

a) For using a silica gel-based column: 0,25 mol/L phosphate buffer solution (pH7,0) (see 5.10.1);

b) For using a polymer-based column: 1 mol/L phosphate buffer solution (pH2,9) (see 5.10.3).

NOTE It has been confirmed that sample solutions will remain stable for at least 3 days when kept at 10 °C.

8.6 Measurement

8.6.1 Setting of operating conditions

In accordance with the manufacturer's instructions, set the HPLC conditions as follows:

NOTE 1 In the case the mobile phase contains a large amount of potassium ions, it reacts with perchlorate ions contained in the sample solution and forms a precipitate, which interferes with the measurement by HPLC. Accordingly, in this document, sodium salts are used to prepare the mobile phase.

a) For using a silica gel-based column:

   1) Mobile phase: 0,05 mol/L phosphate buffer solution (pH7,0) (see 5.10.2);
   2) Column temperature: 40 °C;
   3) Measurement wavelength: 260 nm;
   4) Injection volume: 10 µL.

NOTE 2 In the interlaboratory tests described in Annex A, flow rate was set to 1,0 mL/min.

b) For using a polymer-based column:

   1) Mobile phase: 0,2 mol/L phosphate buffer solution (pH2,9) (see 5.10.4);
   2) Column temperature: 40 °C;
   3) Measurement wavelength: 260 nm;
   4) Injection volume: 20 µl.

NOTE 3 In the interlaboratory tests described in Annex A, flow rate was set to 0,6 mL/min.

8.6.2 HPLC analysis

Stabilize the entire system appropriately. Confirm that the fluctuation of base line gives no hindrance for measurement of the ATP-related compounds by a blank run under the specified condition (see 8.6.1). Then inject a series of standard working solutions and the sample solutions (see 8.5) onto the column. The order of injections should be random.

8.6.3 Identification

Identify the individual ATP-related compounds peaks in the sample chromatograph by comparing retention times with those obtained from the standard solutions under the same chromatographic conditions (see 8.6.1).

NOTE Typical HPLC chromatograms of ATP related compounds are given in Annex C.

9 Calculation

9.1 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 specified in JIS K 0124.

9.2 Calculation of K-value

9.2.1 Obtain the areas of the ATP-related compounds in each of a series of standard working solutions. Construct linear calibration graphs of each ATP-related compound, for each standard concentration against the peak area of each ATP-related compound obtained from the data collection/integration system. The correlation coefficients of the linear calibration are required to be 0,998 or more.

NOTE In the interlaboratory tests described in Annex A, with reference to the content of ATP-related compounds in general Osteichthyes, the standard working solutions with the following concentrations were used for each ATP-related compound:

— for ATP: about 0,8 μmol/L, about 3,2 μmol/L, about 8 μmol/L, about 32 μmol/L, about 160 μmol/L and about 320 μmol/L;

— for ADP: about 0,8 μmol/L, about 3,2 μmol/L, about 8 μmol/L, about 32 μmol/L, and about 160 μmol/L;

— for AMP: about 0,8 μmol/L, about 3,2 μmol/L, about 8 μmol/L, and about 32 μmol/L;

— for IMP: about 0,8 μmol/L, about 3,2 μmol/L, about 8 μmol/L, about 32 μmol/L, about 160 μmol/L, about 320 μmol/L, and about 480 μmol/L;

— for HxR: about 0,8 μmol/L, about 3,2 μmol/L, about 8 μmol/L, about 32 μmol/L, about 160 μmol/L and about 320 μmol/L;

— for Hx: about 0,8 μmol/L, about 3,2 μmol/L, about 8 μmol/L, about 32 μmol/L, about 160 μmol/L and about 320 μmol/L.

9.2.2 Calculate the concentrations of the ATP-related compounds from the peak area of each sample solution by the calibration curves. The content of ATP-related compound X in the test sample, b_X, is given by the following formula:

$$b_X = c_{X,sp} \times \frac{V_3 \times 10^{-3}}{m} \times \frac{V_4 + V_5}{V_4} \quad (2)$$

where

b_X is the content of ATP related compound X in the test sample (μmol/g);

$c_{X,sp}$ is the concentration of X in the sample solution (μmol/L);

V_3 is the constant volume (mL) at filling up in 8.3.2;

m is the mass (g) of the test sample;

V_4 is the volume (μL) of the filtrate in 8.5;

V_5 is the volume (μL) of the phosphate buffer in 8.5.

NOTE In the interlaboratory tests described in Annex A, V_3 was 50 mL, V_4 was 1000 μL, and V_5 was 250 μL.

9.2.3 Calculate the K-value from the contents of ATP-related compounds in the test sample by the following formula:

$$\text{K-value} = \frac{b_E + b_F}{b_A + b_B + b_C + b_D + b_E + b_F} \times 100 \quad (3)$$

where

b_A is the ATP content in the test sample (μmol/g);

b_B is the ADP content in the test sample (μmol/g);

b_C is the AMP content in the test sample (μmol/g);

b_D is the IMP content in the test sample (μmol/g);

b_E is the HxR content in the test sample (μmol/g);

b_F is the Hx content in the test sample (μmol/g);

9.3 Expression of results

Express the results to two significant figures. Describe the unit (%) with them, as needed.

10 Precision

10.1 Interlaboratory test

The interlaboratory test was carried out to determine the precision of the test method, and the results are summarized in Annex A. The values derived from this interlaboratory test can be inapplicable to the content ranges other than the given one (6,12 % to 83,4 %).

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, is expected in not more than 5 % of cases to be greater than the repeatability limit (r) values [5] shown in Table A.1 on average as long as the specified operation is correctly done [6].

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, is expected in not more than 5 % of cases to be greater than the reproducibility limit (R) values [5] given in Table A.1 on average as long as the specified operation is correctly done [6].

11 Quality control

The laboratory shall have internal quality control procedures for tests.

12 Test report

The test report shall include at least the following information:

a) the title or the reference number of this document;
b) every detail to identify the test sample;
c) the date of the test;
d) the results of the test.

Annex A
(informative)

Results of interlaboratory tests

Interlaboratory tests were carried out in accordance with IUPAC protocol [7] in 2021 in Japan, and gave the statistical results shown in Table A.1. The ordinary meats of lateral muscles of commercially available fresh Osteichthyes were collected, chopped, frozen in liquid nitrogen to suppress deterioration, and crushed while frozen. The crushed materials were subdivided into about 2,0 g, which is the amount to be used for one test, and were used as a test sample, after the homogeneity [8] was confirmed. The test samples were stored at -80 °C until sent to the participating laboratories. The experimental protocol, the stock standard solutions of ATP-related compounds and test samples were supplied to the participating laboratories by the Hakodate Regional Industry Promotion Organization, Public Interest Incorporated Foundation, the organizer of the interlaboratory tests, and the mass of the test sample were notified. The temperature of the test samples was maintained below -20 °C during transportation to each laboratory and until the start of the test. Within 4 days of receipt, each laboratory tested a total of 10 test samples (5 pairs of blind duplicates) according to the experimental protocol. In order to avoid deterioration of the samples due to thawing, each laboratory conducted the test using the entire amount of the test samples in the frozen state, and used the mass of the samples notified by the organizer to calculate the K-value.

Table A.1 — Precision data

Sample identification	**1**	**2**	**3**	**4**	**5**
Number of participating laboratories	11	11	11	11	11
Number of accepted test results	11	10	9	10	11
Mean value, %(K-value)	6,12	10,08	26,78	39,25	83,4
Repeatability standard deviation s_r, %(K-value)	0,10	0,11	0,05	0,11	0,2
Repeatability relative standard deviation RSD_r, %	1,6	1,1	0,18	0,28	0,2
Repeatability limit r (r = 2,8 s_r), %(K-value)	0,27	0,31	0,14	0,31	0,6
Reproducibility standard deviation s_R, %(K-value)	0,21	0,23	0,45	0,50	1,2
Reproducibility relative standard deviation RSD_R, %	3,5	2,3	1,7	1,3	1,4
Reproducibility limit R (R = 2,8 s_R), %(K-value)	0,60	0,65	1,3	1,4	3,3

Key to samples:

1 Japanese flounder(*Paralichthys olivaceus*), wild caught.

2 Yellowtail(*Seriola quinqueradiata*) , wild caught.

3 Yellowtail(*Seriola quinqueradiata*), farmed.

4 Pacific mackerel(*Scomber japonicus*), wild caught.

5 Atlantic salmon(*Salmo salar*), farmed.

Annex B
(informative)

Results of studies on the pH of HxR standard solutions for absorbance measurement

Regarding the molar absorption coefficient of ATP-related compounds, the values in pH7,0 phosphate buffer solution are used for ATP, ADP, AMP, IMP, and Hx, and the value in pH6,0 phosphate buffer solution is used for HxR [3]. Studies to change the pH of absorbance measurement solution of HxR to pH7,0 as well as other ATP-related compounds, were conducted to reduce workload for user of this document.

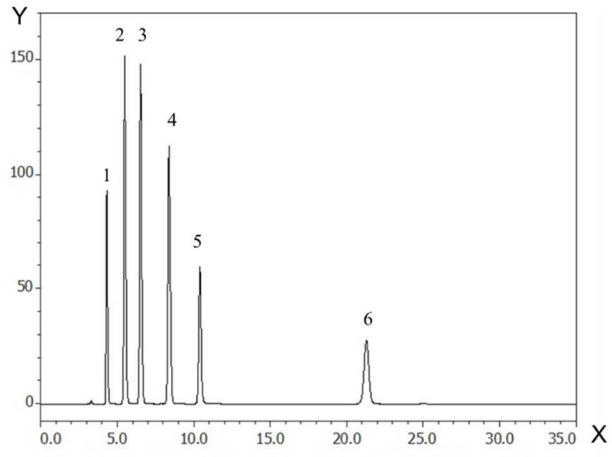
The evaluation results of the absorbance of the HxR solution due to the difference between pH6,0 and pH7,0 of the solvent are shown in Table B.1.

Table B.1 — Absorbance of HxR solutions at pH6,0 and pH7,0

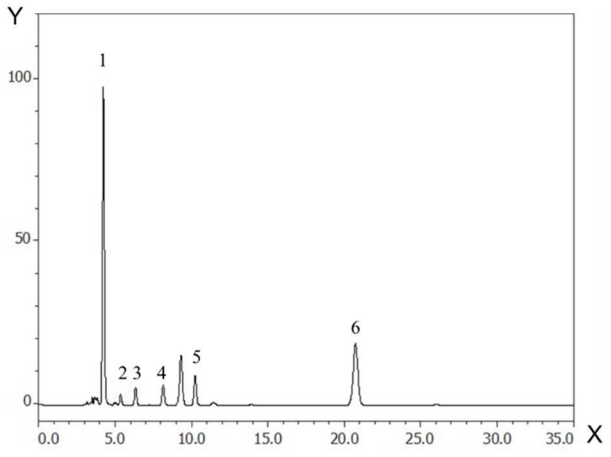
Replicate	Absorbance	
	pH6,0	pH7,0
1	0,617	0,619
2	0,617	0,616
3	0,617	0,617
4	0,617	0,619
5	0,618	0,617
Mean value	0,617	0,618
NOTE Significant difference could not found in the absorbance between at pH6,0 and at pH7,0 by Welch's t-test (two-tail)(significance level 5 %).		

Annex C
(informative)

Typical HPLC chromatograms



a) Standard working solution(ATP-related compounds, each with a concentration of about 160 μmol/L)



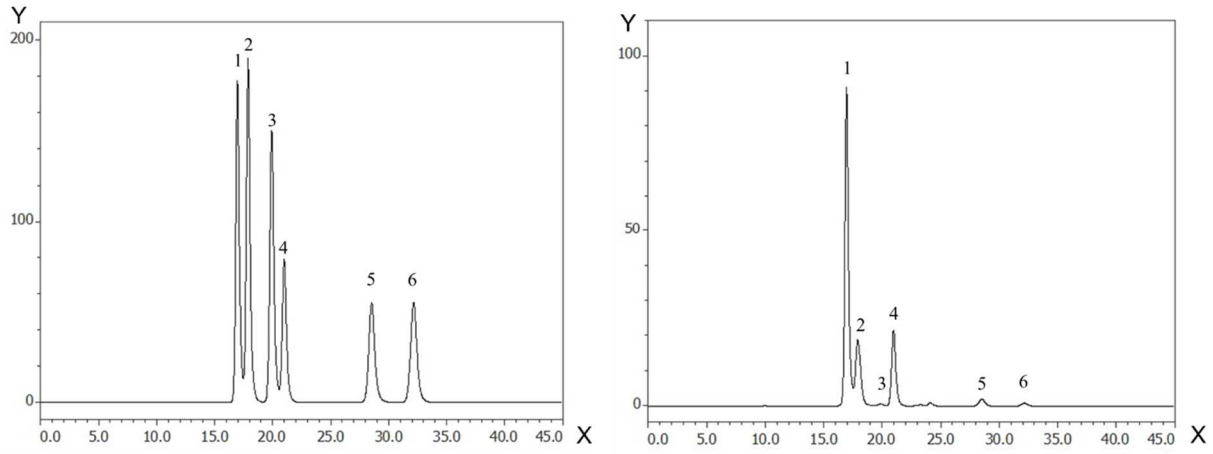
b) Sample solution[Bluefin tuna(*Thunnus orientalis*)]

Key

X	Retention time, min	3	ADP
Y	Response, mV	4	AMP
1	IMP	5	Hx
2	ATP	6	HxR

NOTE HPLC operating conditions are in accordance with 8.6.1a) and CAPCELL PAK ADME-HR™ was used as the column. This information is given for the convenience of users of this document and does not constitute an endorsement of this product.

Figure C.1 — Examples of the chromatograms using the silica gel-based column



a) Standard working solution(ATP-related compounds, each with a concentration of about 160 μmol/L)

b) Sample solution[Japanese flounder(*Paralichthys olivaceus*)]

Key

X	Retention time, min	3	AMP
Y	Response, mV	4	IMP
1	ATP	5	HxR
2	ADP	6	Hx

NOTE HPLC operating conditions are in accordance with 8.6.1b) and Asahipak® GS-320 HQ was used as the column. This information is given for the convenience of users of this document and does not constitute an endorsement of this product.

Figure C.2 — Examples of the chromatograms using the polymer-based column

Bibliography

[1] Saito, T., et al., A new method for estimating the freshness of fish. Nippon Suisan Gakkaishi, 1959, 24(9), pp. 749-750

NOTE The K-value calculation formula in the referenced document was referred to for calculating the K-value.

[2] Lee, EH., et al., High performance liquid chromatographic determination of K value as an index of freshness of fish. Nippon Suisan Gakkaishi, 1982, 48(2), pp. 255

NOTE The analysis method in the referenced document was referred to for the method of calculating K-value using the measured value of HPLC.

[3] National Academy of Science, Specifications and Criteria for Biochemical Compounds. 3rd Edition., Washington D.C..1972

NOTE Molar absorption coefficient of the ATP-related compounds in referenced document was referred to for calculating the concentration of the ATP-related compounds standard solution using the measured value of spectrophotometer.

[4] HU, Y., et al., Development of simplified method for extracting ATP-related compounds from fish meat. Nippon Suisan Gakkaishi, 2013, 79(2), pp. 219-225

NOTE "Results, and the neutralization conditions of the extract" of the referenced document was referred to for the pH adjustment of the suspended extract.

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

NOTE Section 4 "Determination of limits" of the referenced document was referred to for the calculation of the repeatability limit and the reproducibility limit.

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

NOTE Section 7.1.5 of the referenced document was referred to for the expression of the repeatability limit and the reproducibility limit.

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

[8] ISO 13528:2005, Statistical methods for use in proficiency testing by interlaboratory comparisons

NOTE Appendix B of the referenced document was referred to for the method to confirm the homogeneity.