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Application News

No. L536

High Performance Liquid Chromatography

Analysis of Nucleic Acid Related Substances in Fish Meat and Automatic Calculation of Freshness (K Value) Using Multi-Data Report Function

Compared to the muscle tissue of livestock animals, the flesh of fish or shellfish is known to decay more rapidly due to its softness and high water content. Accurately determining the freshness of these fish and shellfish is extremely important in terms of food safety and security.

Changes in the concentration of ATP (adenosine triphosphate) in animals' muscles are widely used as an indicator of muscle freshness. Moreover, the so-called K-value is often used as a method for the freshness evaluation of fish.

In recent years, cases have been reported of allergies developing from histamine food poisoning. When a red fish such as tuna decays, histamine (a metabolite of histidine, which is a kind of amino acid) accumulates in high concentrations. Although it is possible to detect histamine using HPLC, the pretreatment (derivatization) is complicated, and a large system is required for automatic pretreatment. Therefore, it is useful to be able to measure the state of decay simply by analyzing ATP-related compounds using the Nexera™ LC system with a simple configuration.

In this report, we use the K-value to determine the freshness of tuna from HPLC analysis and demonstrate the use of the multi-data report function.

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Analysis of ATP-Related Compounds

A standard solution (10 µmol/L) of ATP-related compounds (hypoxanthine (Hx), inosine (HxR), IMP, AMP, ADP, ATP) *1 was analyzed. The chromatogram is shown in Fig. 1, and the analytical conditions are shown in Table 1.

A Shim-pack GIST C18 AQ column was used for chromatographic separation. This column shows high performance and durability even when a mobile phase with a composition close to 100 % aqueous solution is used.

Nucleobases and nucleotides represented by ATP-related substances are often separated by isocratic elution using a phosphate buffer, therefore it takes long time to analyze these compounds.

In this paper, the separation of these compounds was accomplished by gradient elution using mobile phase added ion pair reagent in order to retain a compound having phosphate group, and analytical time was shortened. In addition, in order to prevent pressure increase at the column due to clogging of contaminating compounds in sample, a column washing step was added.

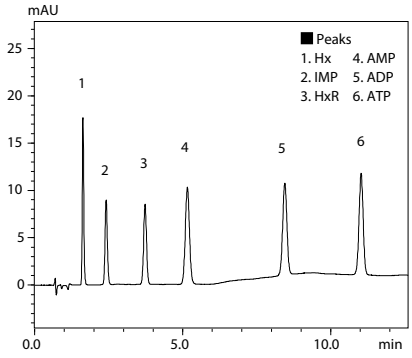

Fig. 1 Standard Solution of ATP-Related Compounds

Table 1 Analytical Conditions

Column	Shim-pack™ GIST 3 µm C18 AQ (100 mm L. × 3.0 mm I.D., 3 µm)
Flow rate	: 0.8 mL/min
Mobile phase *2	: A) Water/ acetonitrile=100/1 (v/v) containing 0.15 mol/L phosphoric acid, 0.225 mol/L triethylamine B) Water/ acetonitrile=80/20 (v/v) containing 0.15 mol/L phosphoric acid, 0.225 mol/L triethylamine
Time Program	: 0 %B (0-3.5 min) → 12 %B (11 min) → 100 %B (11.01- 18 min) → 0 %B (18.01-28 min)
Column temp.	: 30 °C
Injection volume	: 10 µL
Detection	: PDA 260 nm

*1: IMP : Inosine 5'-monophosphate, AMP : Adenosine 5'-monophosphate, ADP : Adenosine 5'-diphosphate, ATP : Adenosine 5'-triphosphate

*2: Phosphoric acid: 10.2 mL, Triethylamine: 31 mL } → mixture of Water/ Acetonitrile 1 L

Reproducibility

Table 2 shows the coefficient of variation (% RSD) of retention time and peak area over 6 repeats for each ATP-related compound. The coefficient of variation was <1% for both retention time and area value of every compound in the analysis.

Table 2 Coefficient of Variation (% RSD) (n=6)

Compounds	Retention time	Area
Hx	0.14	0.33
IMP	0.00	0.21
HxR	0.09	0.20
AMP	0.04	0.32
ADP	0.05	0.50
ATP	0.05	0.68

Estimation of the Freshness of Fish

Both fish and shellfish use ATP as the energy source in their muscle tissue. However, ATP is no longer synthesized after death, so the ATP concentration in the muscle tissue progressively decreases via enzymatic degradation in the following path: ATP → ADP → AMP → IMP → HxR → Hx.

The K-value of fish and shellfish muscles is defined as the percentage of the sum of the compounds that do not contain phosphoric acid (hypoxanthine, inosine) over the total amount of ATP-related components.

$$K = \frac{Hx + HxR}{Hx + HxR + IMP + AMP + ADP + ATP} \times 100$$

The above formula shows that smaller K-value is directly related to the freshness of the muscle tissue. The K-value is affected by various factors (storage temperature, time in storage etc.).

Calibration Curve

Calibration curves were prepared for the 6 ATP-related compounds. Good linearity was obtained with $R^2 = 0.9999$ or greater for each component (Table 3).

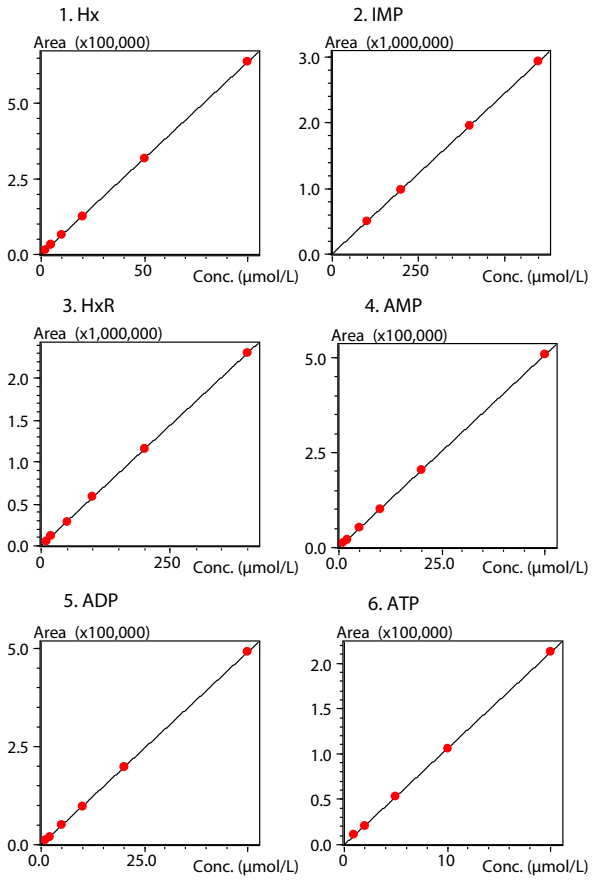

Fig. 2 Calibration Curves for ATP-Related Compounds

Table 3 Calibration Curve Concentration Range (µmol/L) and R^2 Value for Each ATP-Related Compound

Compounds	Conc. Range (µmol/L)	R^2
Hx	2-100	0.9999972
IMP	100-600	0.9999905
HxR	10-400	0.9999822
AMP	1-50	0.9999917
ADP	1-50	0.9999960
ATP	1-20	0.9999931

Sample Preparation

For our samples, we used commercially-available fresh albacore tuna and yellowfin tuna thawed from frozen. The sample preparation protocol was previously described in "Comparison of freshness changes in fresh and frozen black marlin via K-value", 2012 [1].

The homogenized sample was extracted with 10 % perchloric acid and then extracted twice with 5 % perchloric acid. The supernatant of the three extracts was ice-cooled, neutralized with KOH solution (10 N, 1 N, 0.1 N), and filtered prior to HPLC injection (Fig. 3).

Tuna Freshness Measurement

We analyzed samples immediately after purchase and after 1 to 3 days in order to measure changes in K-value. Fig. 4 shows the chromatograms for the fresh albacore tuna, and Fig. 5 shows the chromatograms for the yellowfin tuna. The lower diagrams in Fig. 4 and 5 show enlarged view.

Table 4 shows the change in K-value over time and the quantitative value (µmol/L) of each component.

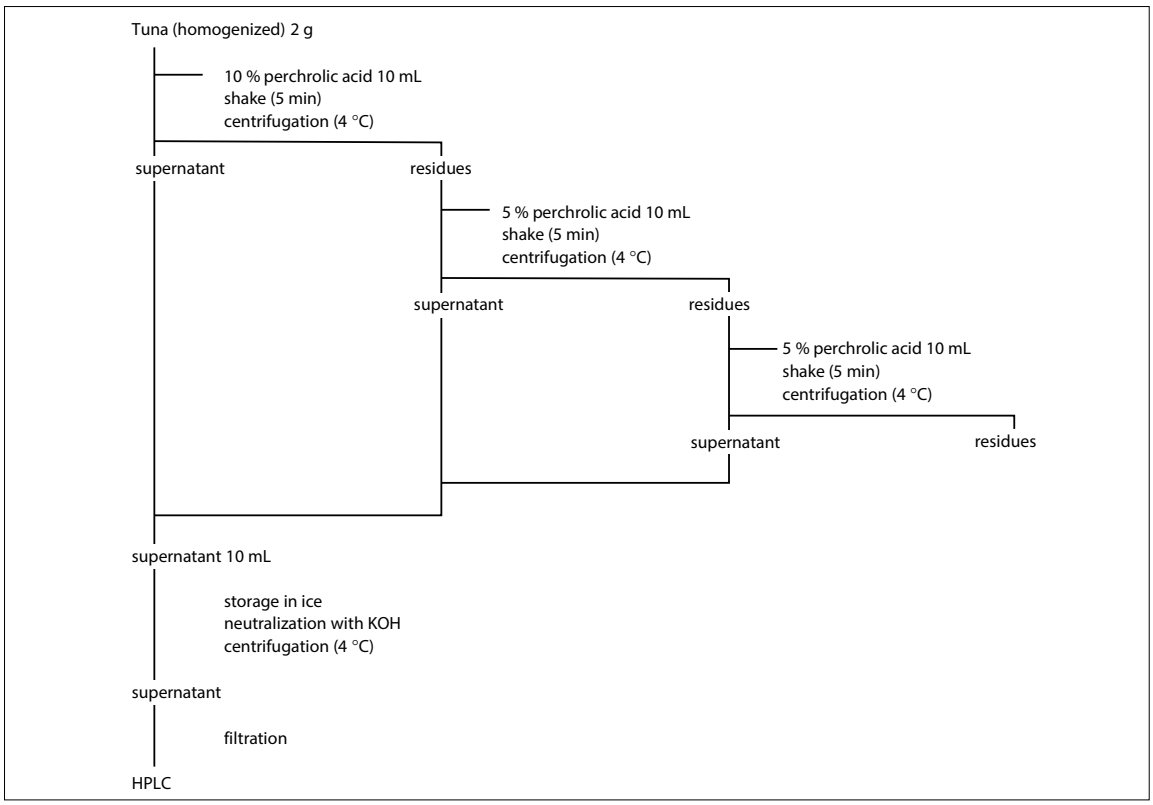

Fig. 3 Sample Preparation Procedure

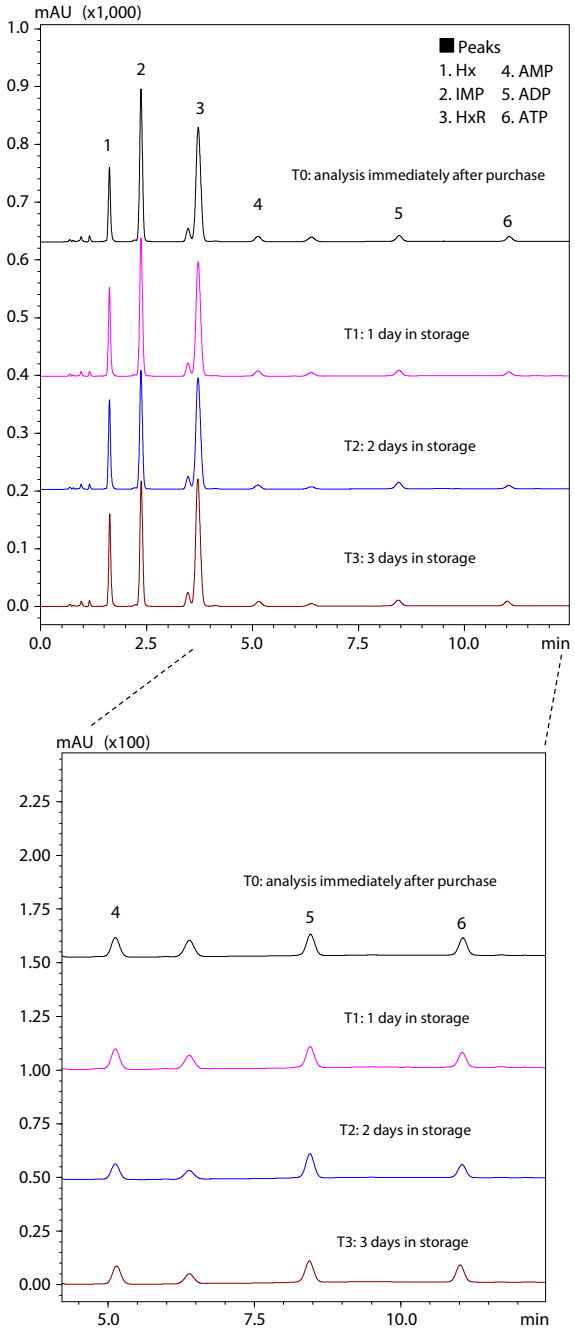

Fig. 4 Chromatograms of Fresh Albacore Tuna

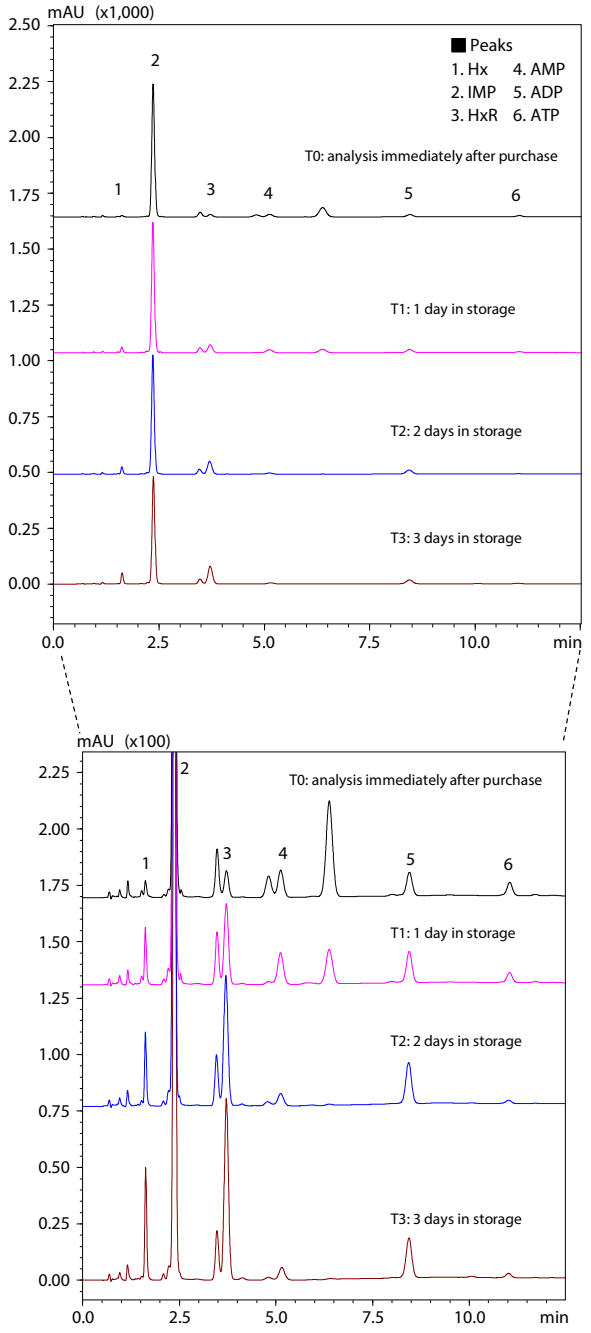

Fig. 5 Chromatograms of Yellowfin Tuna Thawed from Frozen

Table 4 Change in K-Value and Quantitative Value (μmol/L) for Each Component.

Albacore Tuna

days	K value (%)	Quantitative value (μmol/L)					
		Hx	IMP	HxR	AMP	ADP	ATP
0	53.8	68.970	252.281	256.739	9.052	10.552	7.882
1	56.9	83.502	229.872	254.640	9.286	9.962	6.728
2	60.2	83.825	195.520	249.527	7.197	12.054	5.841
3	61.5	87.607	203.348	279.969	8.561	10.889	7.659

Yellowfin Tuna

days	K value (%)	Quantitative value (μmol/L)					
		Hx	IMP	HxR	AMP	ADP	ATP
0	2.5	3.619	582.971	12.103	10.156	10.657	5.724
1	8.7	12.794	559.034	43.472	13.641	14.310	4.413
2	14.5	17.020	496.849	71.427	4.992	19.459	1.574
3	20.9	26.589	451.304	99.401	5.299	18.558	1.926

Durability of the Column

The durability of the Shim-pack GIST C18 AQ column used for this report was evaluated. It was confirmed that column loading pressure (maximum pressure) does not rise even after 300 injections of tuna samples.

The theoretical plate number and symmetry coefficient before the 1st injection and after 300 injections of tuna sample were compared. There were no significant deterioration for both parameters (less than 10 %).

Table 5 summarizes the results for column load pressure (maximum pressure), theoretical plate number, and symmetry coefficient, comparing the status before the first injection and after the 300th injection.

Table 5 Reduction Rate of Each Parameter Before Actual Sample Injection and After 300 Injections.

Pmax. (MPa)

	Number of Injections		Decreasing rate (%)
	0	After 300	
Pmax.	22.15	22.15	0

Theoretical plate, N

	Number of Injections		Decreasing rate (%)
	0	After 300	
Hx	7692	7413	3.63
IMP	5692	5256	7.66
HxR	7679	7416	3.42
AMP	7057	6410	9.17
ADP	17048	16408	3.75
ATP	31533	28970	8.13

Symmetry

	Number of Injections		Deterioration rate (%)
	0	After 300	
Hx	1.165	1.163	-0.17
IMP	1.021	1.097	7.44
HxR	0.99	1.036	4.65
AMP	0.997	1.054	5.72
ADP	0.97	0.994	2.47
ATP	0.971	0.996	2.57

Use of Multi-Data Report Function *3

Multi-data report is one of the reporting functions that automatically inserts analytical results into a spreadsheet file. As soon as the batch analysis is finished, the results are automatically updated and reported. This function can be used also for data post-processing, generating a report from previously acquired data.

In this case, we created a multi-data report automatically from the tuna K-value measurement results (Fig. 6). By automatically generating a graph, it was possible to monitor the change in freshness of the tuna samples. The K-value of the fresh albacore tuna was 53.8 % on the day of purchase (T0). However, after two days of refrigerated storage (T48h), the K-value increased to >60 % due to the decay process. The thawed yellowfin tuna had a K-value of 2.5 % on the day of purchase (T0), suitable to be eaten raw. However, after three days of refrigerated storage (T72h), the K-value was >20 %, and no more suitable for raw consumption.

*3: The multi-data report function is supported by LabSolutions™ DB/CS

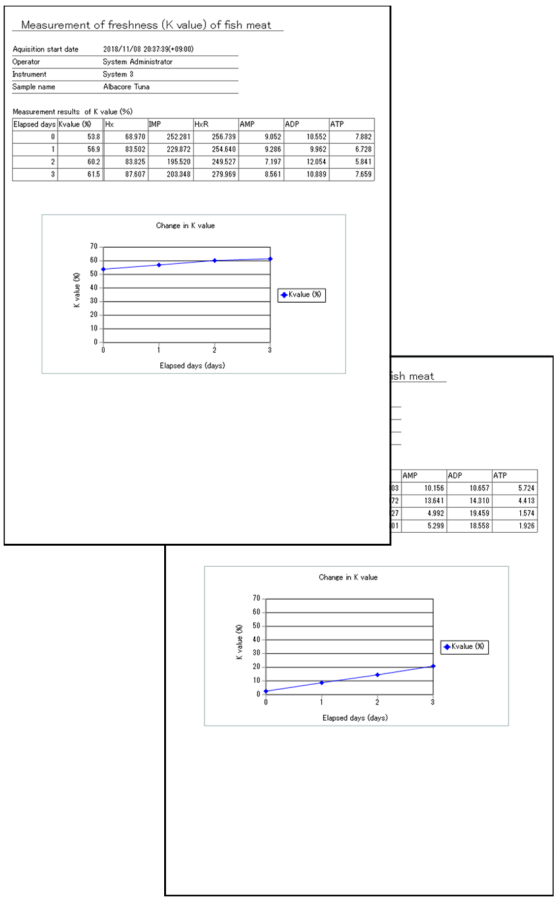

Fig. 6 Multi-Data Report Showing the Results of the Tuna Freshness Measurements

[References]
1) Usui Shigeru, Watanabe Etsuo, "Comparison of freshness changes in fresh and frozen black marlin via K-value", 2012. (Japanese only)

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