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Application News

No. L554A

High Performance Liquid Chromatography

Quick Estimation of the Freshness and the Level of Putrefaction in Fish Meat Using Nexera™ Dual Injection System

–Simultaneous Analysis of ATP-Related Compounds, Histamine and Amino Acids in Fish Meat–

The muscles in fish meat are easily spoiled due to its rough structure and large water content compared to those in farm-animal meat. Consequently, the accurate estimation of fish meat freshness is very important to maintain the safety of food. The variance of adenosine triphosphate (ATP), which is a source of energy for muscles of animals is commonly used as an index of animal meat freshness. In case of numerical estimation of fish meat freshness, the unique K value, related to ATP decomposition is commonly employed in Japan.

On the other hand, it is reported that histamine, one of the putrefactive amines, induces allergy through a food intoxication condition. Histamine (one of the metabolites of histidine) is highly accumulated in red fish meat in the putrefactive process.

Once histamine is generated, it is not possible to prevent food intoxication because histamine is resistant to heat and cannot be removed during cooking process. To address this issue, the Codex Alimentarius Commission (Codex), European countries, and elsewhere have established the official limitation for acceptable levels of histamine concentration.

Application News No. L536 introduced determination of the K value in fish meat and the resulting multi-data report on the changes in the freshness over time. This article introduces simultaneous determination of the K value as an indicator of freshness and histamine as an indicator of the level of putrefaction using the Nexera™ dual injection system. Under the analytical conditions described in this article, amino acids and nucleic acids known as nutritional compounds, including *umami* taste, contained in fish meat were able to be determined simultaneously as well.

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Dual Injection System Capable of Two Different Analyses Simultaneously

Generally, ATP-related compounds and histamine are not determined simultaneously so that two independent HPLC analyses are essential. Our novel system setup (Nexera dual injection system) affords a significant profit for easily being spoiled samples that have two types of functional compounds requiring different HPLC conditions. In addition, each obtained analysis data is stored as one data file. This facilitates integrate analysis and data management for the same sample.

The employed HPLC setup equipped photodiode array detector (PDA) and fluorescence detector on two independent analytical flow paths within the single HPLC setup. ATP-related compounds and amino acids including histamine were detected with PDA and fluorescence detector respectively.

Target Compounds

The target compounds were thirty-one compounds including six ATP-related compounds, histamine and, twenty-four amino acids (twenty protein compounds and four compounds related to fish meat). These compounds are shown in Table 1.

Table 1 List of ATP-related compounds, histamine and twenty-four amino acids analyzed

ATP-related compounds*1		10	Serine	21	Tyrosine
1	Hx	11	Glutamine	22	Valine
2	IMP	12	Glycine	23	Methionine
3	HxR	13	Histidine	24	Histamine
4	AMP	14	Threonine	25	Cystine
5	ADP	15	β-Alanine	26	Tryptophan
6	ATP	16	Arginine	27	Phenylalanine
Histamine and amino acids		17	Alanine	28	Isoleucine
7	Aspartic acid	18	Taurine	29	Leucine
8	Glutamic acid	19	Anserine	30	Lysine
9	Asparagine	20	Carnosine	31	Proline

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

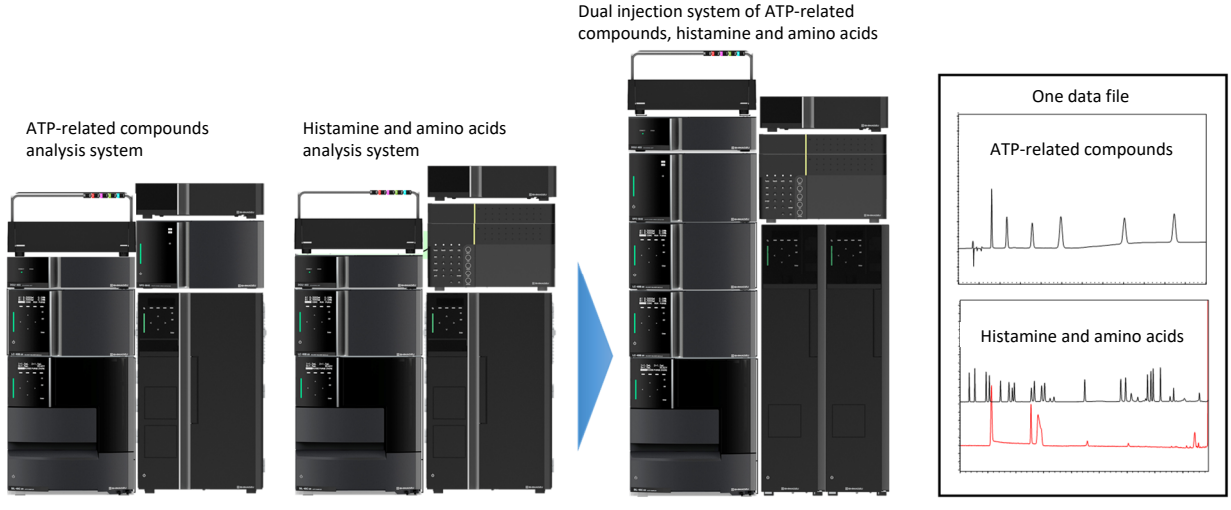

Fig. 1 Each analysis system (shown in left) and dual injection system (shown in right)

Sample Preparations and Analytical Conditions

Tuna samples were prepared through deproteinization, extraction, and pH adjustment (Fig. 2).

Table 2 shows the analytical conditions. Improved analytical stability was obtained due to the gradient elution for ATP-related compounds whereas isocratic elution is employed in ordinary analysis. Amino acids including histamine were automatically derivatized with *o*-phthalaldehyde and 9-fluorenylmethyl chloroformate (Table 3 and 4). Therefore, a complicated manual pretreatment, e.g. dansyl chloride derivatization, is not required so analysis interval including derivatization can be kept constant.

In this article, TORAST™-H Glass Vial, a low-adsorption glass vial was used (Fig. 3). This vials are available in two sizes, 1.5 mL and 150 µL. The 1.5 mL vials were used for the derivatization reagents and the samples before derivatization. The sample was reacted with the derivatization reagents in the 150 µL vial because a mixing in the smaller vial provides good reaction efficiency. Fig. 4 shows the sample setting in the autosampler. The SIL-40 series for the Nexera system can contain three sample racks, and prevent incorrect setting of the samples using independent rack for each specific purpose as shown in Fig. 4.

Table 3 Overview of automatic pre-column derivatization

①	MPA*2 solution 20 µL
②	OPA reagent 20 µL
③	Sample 10 µL
④	Mix
⑤	FMOC reagent 5.0 µL
⑥	Mix
⑦	Injection

*2 Mercaptopropionic acid

Fig. 3 TORAST-H Glass Vial

Table 4 Preparation of derivatization reagents

- MPA solution
  Add 10 µL of 3 - mercaptopropionic acid into 10 mL of 0.1 mol/L borate buffer.
- OPA Reagent
  Add 0.3 mL of ethanol into 10 mg of *o* - phthalaldehyde and dissolve completely. Then add 0.7 mL of 0.1 mol/L borate buffer and 4 mL of ultrapure water.
- FMOC Reagent
  Dissolve 10 mg of 9 - fluorenylmethyl chloroformate into 50 mL of acetonitrile.

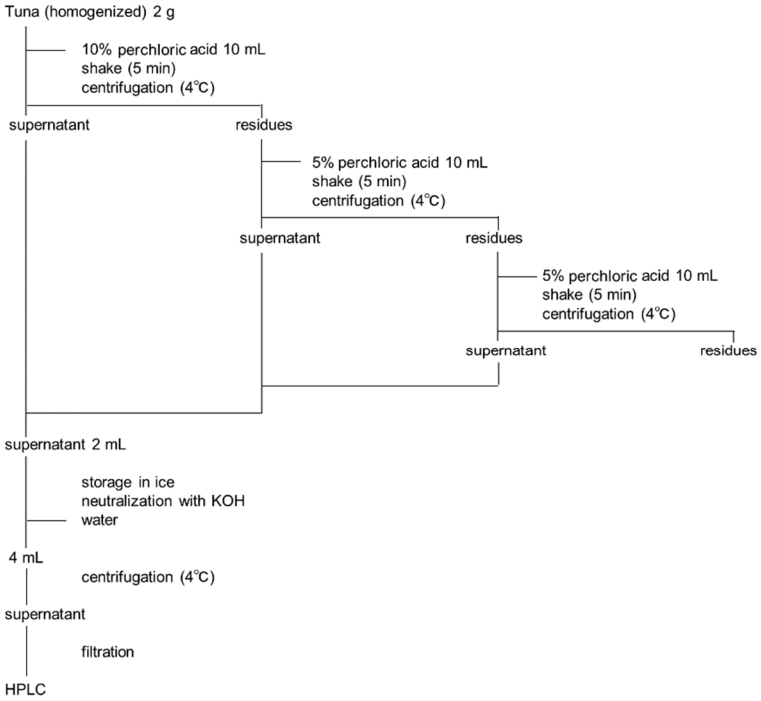

Fig. 2 Sample preparation protocol

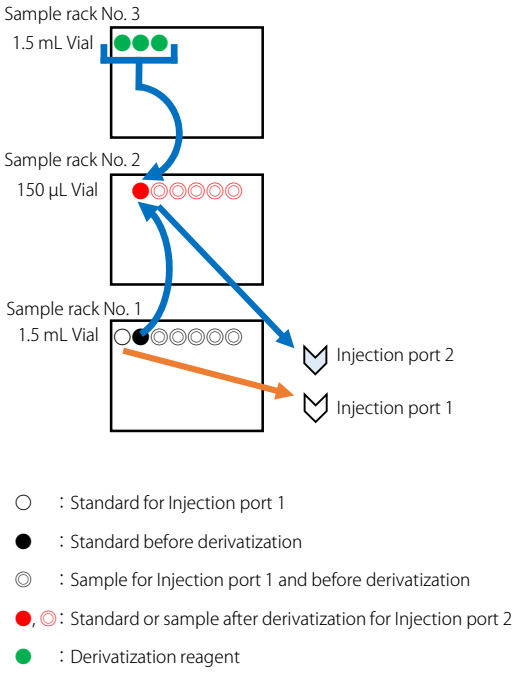

Fig. 4 Sample setting in the autosampler

Table 2 Analytical conditions

System	:	Nexera dual injection system	
		＜ATP-related compounds＞	＜Histamine and amino acids＞
Column	:	Shim-pack™ GIST 3 µm C18 AQ (100 mm L, 3.0 mm I.D., 3 µm)	Shim-pack Velox™ C18 (100 mm L, 3.0 mm I.D., 2.7 µm)
Flow rate	:	0.8 mL/min	0.8 mL/min
Mobile phase	:	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	A) 20 mmol/L (Potassium) phosphate buffer (pH 6.5) B) Acetonitrile/ Methanol/ Water =45/40/15 (v/v/v)
Time program	:	0%B (0-4 min)→12%B (11.5 min)→100%B (11.51-18.5 min)→ 0%B (18.51-32 min)	5%B (0 min)→13%B (8 min)→25%B (15 min)→ 52%B (21.5 min)→100%B (21.51-27.50 min)→ 5%B (27.51-32 min)
Column temp.	:	30 °C	35 °C
Injection volume	:	10 µL	1 µL
Detection	:	PDA 260 nm	FL Ex: 350 nm, Em: 450 nm (Ch1) Ex: 266 nm, Em: 305 nm (Ch2)

Examination of Extraction Solvent and Confirmation of Recovery Rates

A preparation protocol for extracting ATP-related compounds with perchloric acid has been reported for a long time. The extraction efficiency of histamine and amino acids were confirmed in the same way. As the operation blank test, histamine and histidine were extracted with water and perchloric acid. As a result, perchloric acid afforded stable extraction efficiency (Table 5).

Then, six tuna samples were spiked with histamine standard to make concentration of 10 mg/100 g, the threshold limit defined by Codex. 30 minutes later, sample preparation was performed. Table 6 shows the average recovery rates obtained from the results of six samples.

Table 5 Extraction efficiency of histamine and histidine by different extraction solvents

N	Extraction efficiency (%)			
	Histamine		Histidine	
	Water	Perchloric acid	Water	Perchloric acid
1	84.0	97.7	93.8	92.5
2	100.8	95.7	102.1	93.0

Table 6 Recovery rates of histamine (N=6)

N	Recovery rates (%)
1	96.8
2	98.3
3	99.8
4	99.8
5	101.0
6	103.0
Average (%RSD)	99.8 (2.14%)

Calibration Curve

Calibration curves were created for the thirty-one compounds to be analyzed. Good linearity was obtained with r^2 = 0.999 or greater for each compound. The concentration range of calibration curve and r^2 value for each compound are shown in Table 7.

Simultaneous Determination of ATP-related Compounds, Histamine and Amino Acids in Fish Meat, and Determination of K Value and Histamine Concentration

The raw yellowfin tuna under different storage days and temperatures were analyzed to confirm the K values and histamine concentrations. The storage temperature was 4°C and 25°C. In the tuna kept at 4°C for one day, the K value increased slightly by 2.6% and the freshness decreased compared to that immediately after purchase. Histamine was not produced after one day storage at 25 °C, which afforded 25.1% increase of K value.

The raw albacore tuna that had been refrigerated for 6 days afforded a K value of 70.4%, which reached the region called the putrefaction and histamine was detected. The histamine concentration was 2.1 mg/100 g, below the Codex threshold limit (Figure 5 and Table 8).

In addition, histamine was able to be separated from many amino acids such as histidine, alanine, taurine, anserine, carnosine and lysine, that are abundantly contained in fish meat. Table 9 shows the concentrations of ATP-related compounds and amino acids in the tuna samples.

Table 8 Change in K value and concentration of histamine in tuna over time and storage temperature

	days	Temperature (°C)	K value*3 (%)	Histamine (mg/100 g)
Yellowfin Tuna	0	4	36.1	N.D.
	1	4	38.7	N.D.
		25	61.2	N.D.
Albacore Tuna	6	4	70.4	2.1

*3 Definition formula for K value

$$\text{Formula} \quad \frac{Hx+HxR}{Hx+HxR+IMP+AMP+ADP+ATP} \times 100$$

Table 7 Concentration range of calibration curve and r^2 value

	Compound	Conc. range (μmol/L)	r^2		Compound	Conc. range (μmol/L)	r^2
1	Hx	1-300	0.99982	17	Alanine	0.25-100	0.99994
2	IMP	1-300	0.99983	18	Taurine	0.25-100	0.99995
3	HxR	1-300	0.99984	19	Anserine	0.25-100	0.99997
4	AMP	1-300	0.99987	20	Carnosine	0.25-100	0.99995
5	ADP	1-200	0.99998	21	Tyrosine	0.25-50	0.99995
6	ATP	1-200	0.99944	22	Valine	0.25-50	0.99995
7	Aspartic acid	0.25-50	0.99994	23	Methionine	0.25-50	0.99996
8	Glutamic acid	0.25-50	0.99995	24	Histamine	0.25-50	0.99999
9	Asparagine	0.25-50	0.99995	25	Cystine	0.25-25	0.99953
10	Serine	0.25-50	0.99995	26	Tryptophan	0.25-50	0.99993
11	Glutamine	0.25-50	0.99995	27	Phenylalanine	0.25-50	0.99995
12	Glycine	0.25-100	0.99996	28	Isoleucine	0.25-50	0.99995
13	Histidine	0.25-100	0.99968	29	Leucine	0.25-50	0.99996
14	Threonine	0.25-50	0.99994	30	Lysine	0.25-100	0.99995
15	β-Alanine	0.25-50	0.99991	31	Proline	1-25	0.99953
16	Arginine	0.25-50	0.99994				

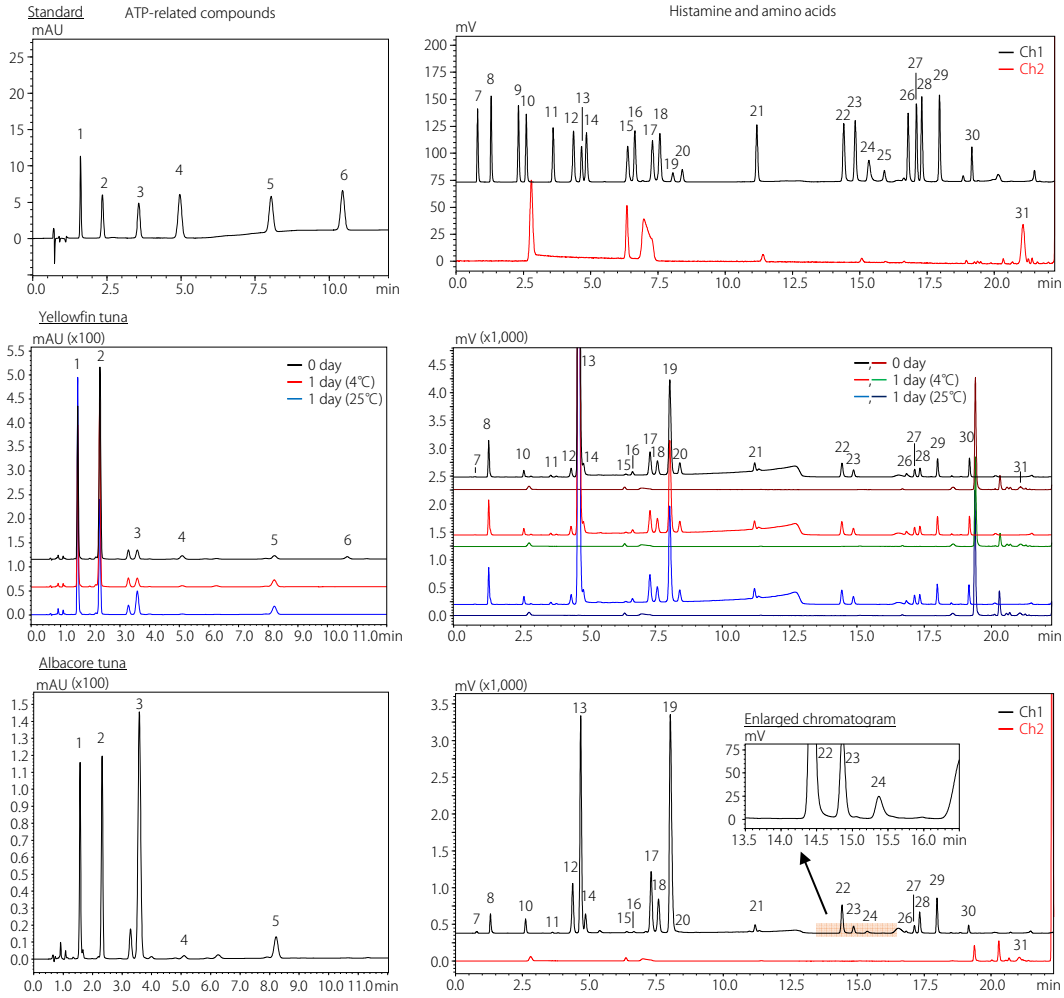

Fig. 5 Chromatograms of the standard solution and tuna sample solution. Peak identification as listed in Table 1.

Table 9 Concentration of ATP-related compounds and amino acids in yellowfin tuna

		Yellowfin Tuna (0 day) (μmol/L)*4
2	IMP	278.9
8	Glutamic acid	46.6
13	Histidine	(2416.0)
17	Alanine	55.7
18	Taurine	27.8
19	Anserine	(1062.5)
20	Carnosine	82.0
22	Valine	23.6
29	Leucine	20.4
30	Lysine	50.7

*4 Values in parentheses are outside the quantification range.

Summary

Simultaneous determination of the K value and histamine concentration was carried out using the Nexera dual injection system. The ATP-related compounds and histamine were able to be extracted by the same sample preparation.

It was shown that K value was changed by storage days and temperature. Histamine was also detected in the spoiled samples showing large K values. Histamine was able to be separated from many amino acids that are abundantly contained in fish meat.

Nexera dual injection system affords a significant profit for food sanitation inspection of easily being spoiled samples that have ATP-related compounds and histamine/ amino acids.

[Reference]

1) Usui Kazushige, Watanabe Etsuo, “Comparison of changes in freshness in fresh and frozen black marlin using the K-value,” Bulletin No. 5 of the Kanagawa Prefectural Fisheries Technology Center, 11-14 (2012)

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