# Evaluating the Freshness of Fish Flesh Using the K Value

## Introduction

The K value is an index for estimating the freshness of fish flesh. The amount of adenosine 5'-triphosphate (ATP) in fish flesh is calculated from a method developed in 1959.[1] Since endogenous enzymes degrade ATP in dead seafoods rapidly, the K value can be a quantitative measurement of seafoods' freshness.

We developed an HPLC method to separate and quantify six ATP related substances in fish flesh extracts. The column used was Shodex Asahipak GS-320 HQ, a multimode column. The GS-320 HQ provides combinations of several separation modes, such as size exclusion, reversed phase, and ion exchange modes, in different degrees by varying the mobile phases. Using this unique feature, nucleic acid and ATP related substances can be separated under isocratic elution methods.

In this technical article, we focused on following three points to show the effectiveness of Shodex Asahipak GS-320HQ for the analysis of ATP related substances.

1. To be able to separate six ATP related substances.
2. To be able to obtain calibration curves, suitable for quantification.
3. The method is not negatively influenced from the sample matrix.

## Experimental

### 1. Sample

The sample was provided by Dr. Takeya Yoshioka, Hokkaido Industrial Technology Center (Hakodate Regional Industry Promotion Organization). There were four different fish; Okhotsk atka mackerel, rainbow trout, Japanese amberjack, and chum salmon. They were shipped and arrived frozen. From each flesh, two test samples were prepared (i.e., total of 8 test samples were prepared). Table 1 summaries the information on the samples used for preparation.

Table 1. Summary of test samples

| Sample No. | Fish | Freshness | Weight (g) |
|---|---|---|---|
| 1 | Okhotsk atka mackerel | High | 1.096 |
| 2 | Okhotsk atka mackerel | High | 1.039 |
| 3 | Rainbow trout | Low | 1.019 |
| 4 | Rainbow trout | Low | 1.058 |
| 5 | Japanese amberjack | Medium | 1.012 |
| 6 | Japanese amberjack | Medium | 1.018 |
| 7 | Chum salmon | High | 1.001 |
| 8 | Chum salmon | High | 1.020 |

### 2. Extraction of ATP related substances

We used the method Hu et al.[2] developed as a reference for the extraction of target substances: 1. Weigh out about 1 g of frozen fish flesh in a 50-mL centrifuge tube (keep the exact weight in a record). 2. Add 20 mL of 5 % perchloric acid to the tube, press and squeeze the flesh with a glass rod for 10 minutes while keeping the tube cooled. 3. Adjust the pH to 3 by adding cooled 1-M potassium hydroxide. 4. Mass up to 50 mL with the HPLC eluent (described later), rest in a refrigerator until the segments settle on the bottom. 5. Filter the supernatant with a 0.45-μm membrane filter and use this as the test sample. The prepared samples were kept in a freezer. The samples were thawed right before the analysis and shaken well before placed on an auto-sampler.

### 3. Preparation of standards

The standards used were Adenosine 5'-triphosphate (ATP) dipotasium hydrate, adenosine 5'-diphosphate (ADP) disodium hydrate, adenosine monophosphate (AMP), disodium 5'-Inosinate (IMP), inosine (HxR), and hypoxanthine (Hx). We prepared 100-mg/L and 500-mg/L stock solutions. The standards other than Hx was dissolved in ultrapure water and Hx was dissolved in 0.1-M sodium hydroxide.[3] The standards with different concentration levels were then prepared by diluting the stock solutions with HPLC eluent. The prepared standards were kept in a freezer. The standards were thawed right before the analysis and shaken well before placed on the auto-sampler.

### 4. HPLC settings

The HPLC system used was SHIMADZU Prominence. The injection volume was 20 μL. The UV cell was set at 40 ºC. The auto-sampler was set at 15 ºC. Details of each analytical condition are mentioned in the result section.

### 5. Calibration curve

The peak areas of each ATP related substances were obtained using automatic integration system (SHIMADZU LabSolutions). The algorism selected was i-PeakFinder. We prepared calibration curves using the least squares method and weighted least squares method for all six substances for comparisons.

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## Results and Discussion

### 1. Optimization of analytical conditions

We optimized the HPLC conditions for the analysis of ATP related substances using Shodex Asahipak GS-320 HQ.

To study the effects of eluent pH, first we tested phosphate buffer by varying its pH. The column temperature was kept at 30 °C. Figure 1 shows the effects of pH on the analytes' retention times. In the pH range 2.5 to 4.5, pH 2.9 provided the best separation balance for the six analytes. The elution sequence of AMP and IMP switched at pH 3.5.

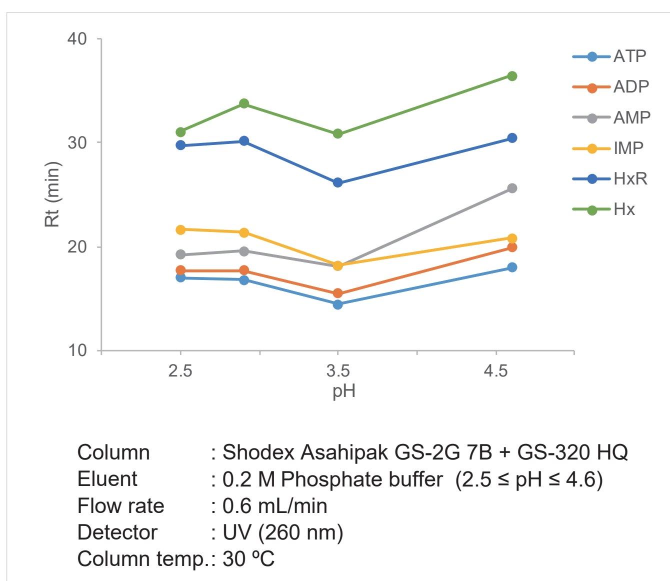


Fig. 1. Effects of phosphate buffer pH on analytes' retention times

Then, we tested acetate buffer as it also has a buffering capability in the low pH range. Figure 2 shows the results.

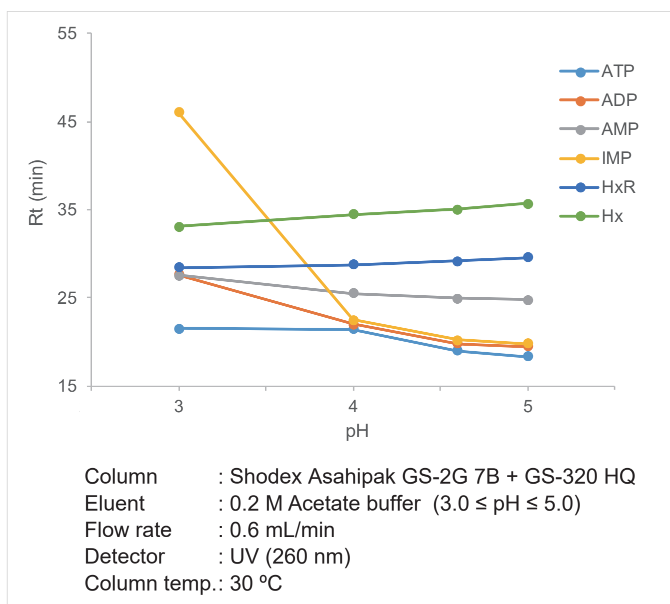


Fig. 2. Effects of acetate buffer pH on analytes' retention times

The separation patterns observed were different from that of phosphate buffer. With the acetate buffer, it was not possible to find a pH that allowed a good separation of all six analytes.

In the next step, we studied the effects of column temperature. The temperature was varied while keeping the phosphate buffer eluent pH at 2.9. Figure 3 shows the effects of column temperature on the analytes' retention times.

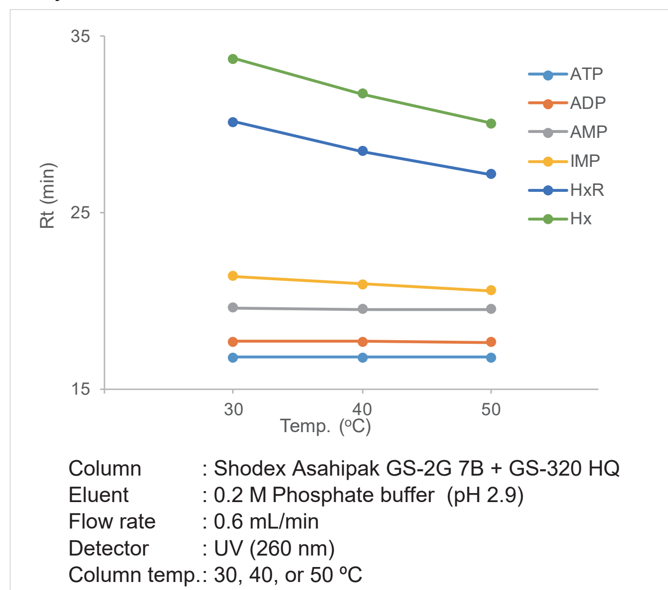


Fig. 3. Effects of column temperature on the analytes' retention times

The retention times of ATP, ADP, and AMP were consistent in the column temperature range 30 to 50 ºC. On the other hand, the retention times of IMP, HxR, and Hx became shorter as the column temperature increased. Considering the separation balance of all analytes and the use of the column oven system during the high temperature seasons, we selected 40 ºC as the optimal.

Therefore, the optimal pH and the column temperature chosen for the analysis of ATP related substances were 2.9 (phosphate buffer) and 40 ºC, respectively.

## 2. Analysis of standards

Figure 4 shows a chromatogram of the six ATP-related-substance standard mixture. All six substances were separated well from each other.

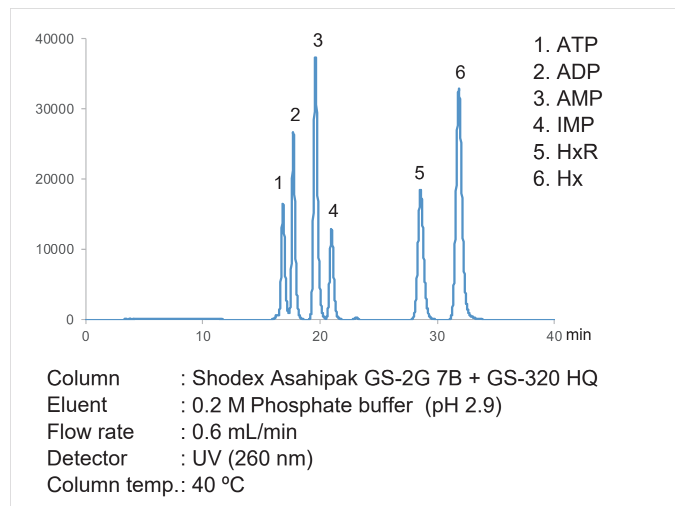


Fig. 4. Chromatograms of 10 mg/L standards

## 3. Analysis of blank solution

A blank solution was prepared by following the “Experimental 2. Extraction of ATP related substances 2 to 5", i.e., extraction solutions were added to an empty centrifuge tube. Figure 5 shows the resulting chromatogram. Two peaks were observed at 20 and 25 minutes. Those peaks did not have the same retention times as any of the six analytes. Thus, most likely they are resulting from the contamination during the extraction procedure.

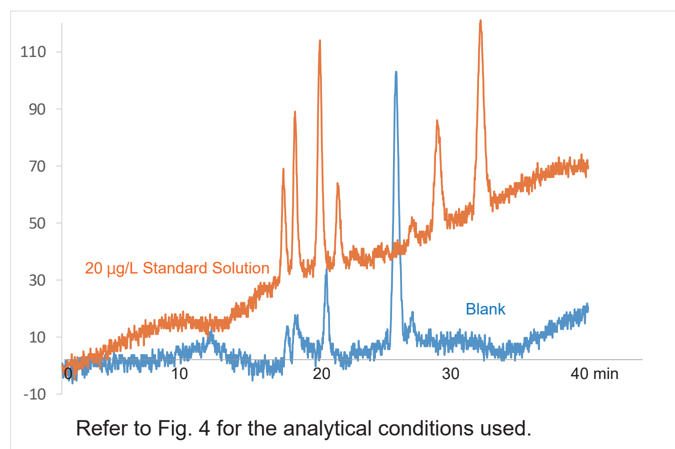


Fig. 5. Chromatograms of blank solution and 20 μg/L standard solution

## 4. Analysis of fish flesh extract

In the Okhotsk atka mackerel extract, no peak of impurity related was found in the positions that could interfere with the six target analytes (Fig.6), and thus did not influence their quantifications. The similar results were obtained for all samples (rainbow trout, Japanese amberjack, and chum salmon).

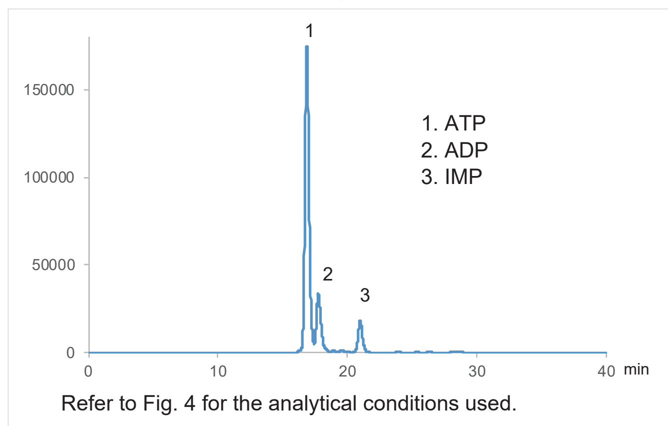


Fig. 6. Chromatogram of an Okhotsk atka mackerel extract

A large ATP peak and small peaks of HxR and Hx, the decomposed products were seen in the Okhotsk atka machkerel extract chromatogram. This demonstrated the high freshness of the sample. The chromatograms of other samples are shown in the appendix at the end of the article.

## 4. Reliability of calibration curves

Figure 7 shows the calibration curve of ATP, using two methods; least squares method and weighted least squares method. Both calibration curves were plotted in log-log graph to compare their accuracies.

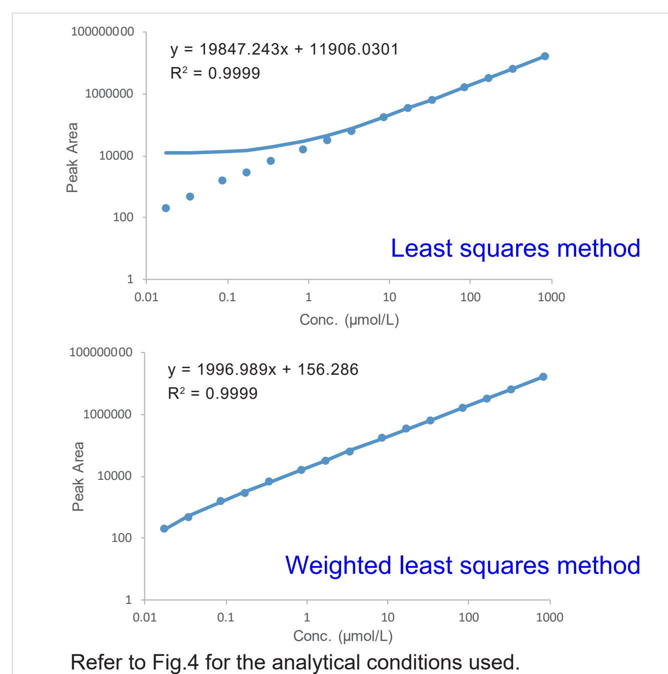


Fig. 7. Calibration curve of ATP

The coefficient of determination ($R^2$) of both methods were close to 1.0. However, in the lower concentration ranges, the values obtained from the least squares curve were different from the actual peak areas. On the other hand, the values obtained from the weighted least squares reflected the actual values more accurately, even in the lower concentration ranges. Therefore, we decided to use the weighted least squares method for quantification.

### 5. Quantification of ATP related substances and the evaluation of freshness using the K value

Table 2 shows the quantified ATP related substances in an Okhotsk atka mackerel extract, using the weighted least squares calibration method.

Table 2. ATP related substances quantified in an Okhotsk atka mackerel extract (Sample No.1)

| | Concentration (μM) |
|---|---|
| ATP | 195 |
| ADP | 37.5 |
| AMP | 1.00 |
| IMP | 29.2 |
| HxR | 1.36 |
| Hx | 0.179 |

The peak value of hypoxanthine (Hx) in the chum salmon, a very fresh sample, was smaller than the peak value of the lowest concentration standard (0.07 μM). Thus, its concentration was calculated using the extrapolated calibration curve. The other values obtained were within the calibration curves of the standards.

A K value can be calculated using the equation 1. The K values of each sample was calculated from the obtained concentrations of ATP related substances (μM) in each extract. Table 3 summaries the result.

$$K = \frac{([HxR] + [Hx]) \times 100}{[ATP] + [ADP] + [AMP] + [IMP] + [HxR] + [Hx]} \quad \text{(Equation 1)}$$

Table 3. Summary of K values

| Sample No. | Fish | Freshness | K (%) |
|---|---|---|---|
| 1 | Okhotsk atka mackerel | HIgh | 0.584 |
| 2 | Okhotsk atka mackerel | High | 0.566 |
| 3 | Rainbow trout | Low | 35.6 |
| 4 | Rainbow trout | Low | 36.2 |
| 5 | Japanese amberjack | Medium | 10.4 |
| 6 | Japanese amberjack | Medium | 10.2 |
| 7 | Chum salmon | High | 0.477* |
| 8 | Chum salmon | High | 0.416* |

* The values for chum salmon should be considered as references, since the concentration of hypoxanthine was calculated using the extrapolated calibration curve.

The smaller the K value, it indicates more fresh the fish is. The results showed that the freshness evaluated from the calculated K values correspond to the actual freshness information received with the samples.

## Conclusions

The results demonstrated that the suitability of Shodex Asahipak GS-320 HQ for the separation of ATP related substances in fish flesh used for the calculation of K value. The method was not affected by the impurities and was successfully applied for the analysis of extracts from four different fish (Okhotsk atka mackerel, rainbow trout, Japanese amberjack, and chum salmon).

## References

1. Tsuneyuki Saito et al. A new method for estimating the fresness of fish. Bulletin of the Japanese Society of Scientific Fisheries, 1959, 24.9: 749.
2. Yaqin Hu et al. Development of simplified method for extracting ATP-related compounds from fish meat. Nippon Suisan Gakkaishi, 2013, 79.2: 219-225.
3. Nancy Cooper et al. Quantification of uric acid, xanthine and hypoxanthine in human serum by HPLC for pharmacodynamic studies. Journal of Chromatography B, 2006, 837.1-2: 1-10.

## Acknowledgment

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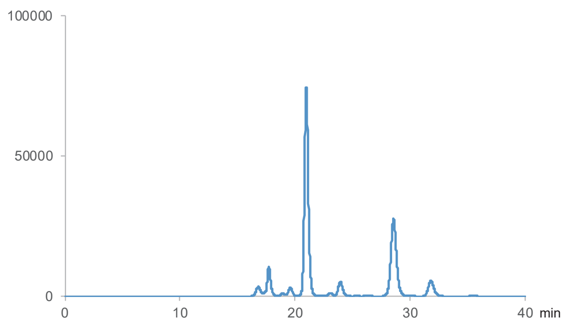
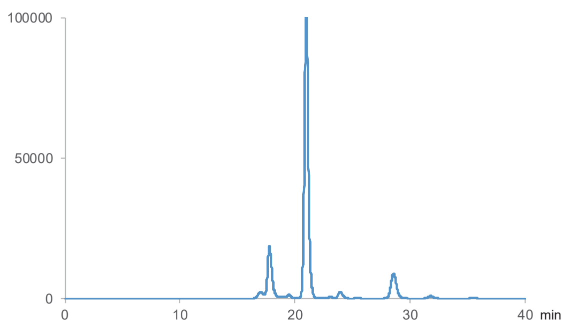
## Appendix

### Chromatograms of samples tested

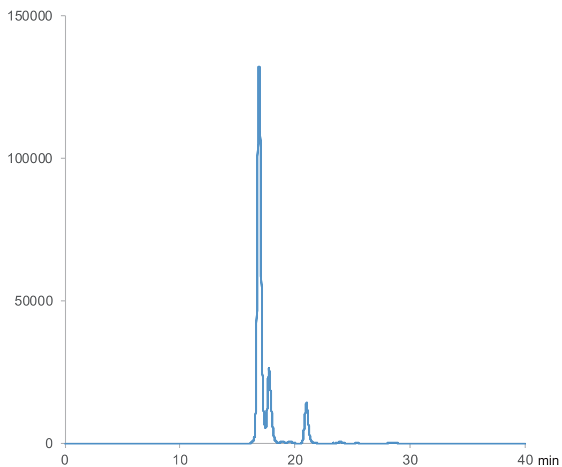
Sample 3: Rainbow trout - Low freshness




Sample 5: Japanese amberjack - Medium freshness




Sample 7: Chum salmon - High freshness

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