

Effects of pH on the Fluorescence Fingerprint of ATP

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Summary

Nowadays, fluorescence spectroscopy has been used as a potential method for nondestructive quality measurement of food materials. Fluorescence fingerprint (FF) of adenosine 5'-triphosphate (ATP) has been observed during the quality assessment of different raw food materials. Although the fluorescence spectra of ATP can be affected by various factors including pH, the details are not clarified yet. Thus, the study attempts to demonstrate the effects of pH (5.0-8.0) on the FF data of ATP standard solutions (10, 5 and 1 $\mu\text{mol/mL}$ for both frozen and non-frozen states). The results of the present study revealed that the strength of the fluorescence signal was influenced by not only the concentration of ATP but also by the pH of samples. The highest fluorescence intensities were observed from the non-frozen ATP solutions at pH 5.0 for each concentration which declined drastically with increasing pH. The majority of frozen ATP samples showed the similar trends of wavelength conditions to get highest fluorescence intensity. Small pH changes affected the intensity and spectral characteristics of FF and it even shifted the peak wavelength conditions. The implementation of this method would be a help to ensure the validity of FF and optimize it as a technique that can be used to verify the effects of pH on many constituents of food.

Keywords: Frozen food, Instrumentation, Fluorescence fingerprint, Nondestructive, ATP, pH

1. Introduction

Fluorescence spectroscopy, a very rapid, highly sensitive, selective and non-invasive technique, has been used in the field of food science and technology. Fluorescence typically occurs from aromatic molecules such as aromatic amino acids, vitamins, nicotinamide adenine dinucleotide (NADH), flavin adenine dinucleotide (FAD), adenosine 5'-triphosphate (ATP), pigments, porphyrin, and lipid oxidation products¹⁾. Thus, fluorescence spectroscopy provides information about the presence of fluorescent molecules and their environment in food materials. For fluorescence detection, there is no longer the need for the expense and a difficulty of handling radioactive tracers for most biochemical measurements as it is highly sensitive to fluorophores²⁾.

Due to its many advantages, the popularity of fluorescence spectroscopy is increasing remarkably. Fluorescence spectroscopy measurement has been widely studied for various food samples such as fish, dairy products, edible oils, meat, fruit, and vegetables³⁾. A food sample is a multi-constituent system as it contains various intrinsic fluorophores. The fluorescent constituents of food can be determined quantitatively as the intensity of the emitted fluorescence depends on the concentration, molar absorptivity, and quantum yield of the molecule⁴⁾. The measurement carried out by using several emission spectra at different excitation wavelengths creates a fluorescence fingerprint (FF)

which is also called excitation–emission matrix (EEM) that covers the total area of fluorescence. Non-invasive freshness estimation of frozen fish by using the EEM of fluorescence spectroscopy has been established very recently in which the K-value was used as a freshness parameter⁵⁾. The optimal dilution ratio for FF of food constituents especially tryptophan and epicatechin has been also reported⁶⁾.

However, the fluorescence spectra can be affected by various factors such as pH, temperature, color, and concentration quenching⁷⁾. Small pH changes can affect the intensity and spectral characteristics of fluorescence and it can even shift the maximum emission wavelength⁸⁾. Moreover, pH is an important parameter in biological science which is related to freshness. Fluorescence from ATP-related compounds has been detected for the quality assessment of both livestock meat⁹⁾ and frozen fish meat¹⁾. However, the effects of pH on the fluorescence spectrum of ATP which is very important for fish freshness assessment have not been investigated so far. The freshness change of livestock/fish meat may occur due to both pH decrease and the degradation of ATP-related compounds simultaneously. Accordingly, clarification of the effect of pH on the FF measurement of ATP-related compounds is an important issue for the application of EEM for freshness evaluation of meat. Thus, an attempt has been taken to know the effects of pH on the FF data of ATP measurement using the standard solution as a model. Furthermore, the FF data of the frozen solutions were also acquired because most of the

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raw foods such as fish meat are necessary to be evaluated the freshness condition in the frozen state.

2. Materials and Methods

2.1 Preparation of buffer and ATP solutions

A 0.1 M buffer solution (Sorensen) of five different pH values (5.0, 5.5, 6.0, 7.0 and 8.0) was prepared by using KH_2PO_4 and Na_2HPO_4 with the aid of a pH meter (LAQUA F-72 pH meter, HORIBA Scientific). Standard ATP, disodium salt was purchased from Oriental Yeast Company Limited (Tokyo, Japan). The pure solution of ATP (20 $\mu\text{mol}/\text{mL}$) was prepared by using ion exchange water. The pure ATP solution was diluted to three concentrations (10, 5 and 1 $\mu\text{mol}/\text{mL}$) and their pH values were adjusted (5.0-8.0) by using the buffer solutions of different pH.

2.2 Preparation of frozen ATP samples

ATP solution was placed in an aluminum tube (2 cm length and diameter), in which one side was covered by plastic film and rapidly frozen by using a mixture of dry ice and hexane. The frozen samples were stored at -60°C for two days and then transferred to a mini refrigerator (Deep freezer, SC-DF25, TWINBIRD) at -30°C . The aluminum tube containing the frozen sample was placed into a cool box filled with the coolant (-30°C) to prevent thawing during FF measurement.

2.3 Instrumental conditions for FF measurement

FFs of the samples were measured using a fluorescence spectrophotometer F-7000 (Hitachi High-Tech Science Corp., Japan) equipped with a xenon lamp (XBO 150 W/4, OSRAM, Germany), an excitation monochromator and an emission monochromator. The data were acquired through FL Solutions software version 4.2 (Hitachi High-Tech Science Corp., Tokyo, Japan). For measuring the ATP solutions, FF was obtained by placing the quartz cell containing the sample solution in a cell holder inside the scanning compartment of the device. The measurement was carried out by setting the emission spectra from 200 to 600 nm at 10 nm intervals, with excitation at every 10 nm from 200 to 600 nm. For both excitation and emission, the slit width was adjusted to 10 nm and the scanning speed was 60,000 nm / min. In contrast, the FFs of the frozen ATP samples were measured by using F-7000 equipped with an external Y-type fibreoptic probe to obtain the fluorescence intensity directly from the inside of the cooling box (-30°C). The excitation and emission wavelength ranges were set from 250 to 600 nm at 10 nm intervals and the slit width was adjusted to 10 nm with a high scanning speed of

60,000 nm / min. The fiber probe was set 2 mm above of the sample to measure FF. The measurement was carried out inside a dark chamber. As the fluorescence intensity changes with the intensity of the excitation light, the detection voltage was adjusted to 400 and 500 V for the solution and frozen samples, respectively. Figure 1 shows the instrumental conditions for measuring both frozen and non-frozen solutions. Measurements were carried out in triplicate for each sample.

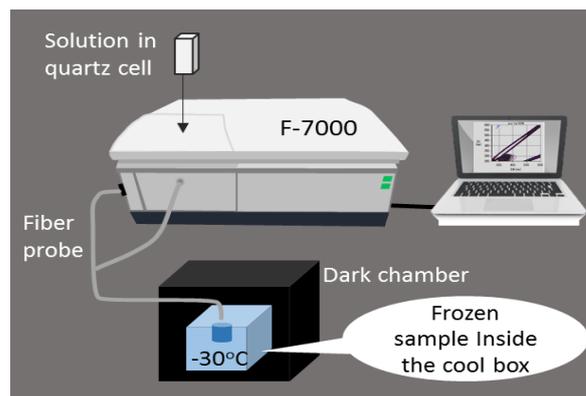


Fig.1. Instrumental conditions of the fluorescence spectrophotometer (F-7000)

2.4 Processing of FF data

The data were processed by using the FL solution software (version 4.2). Each fluorescence fingerprint is a three-dimensional matrix such as the excitation wavelength, the emission wavelength and the fluorescence intensity at the corresponding wavelength condition. Generally, the excitation (λ_{ex}) and the emission (λ_{em}) are plotted by X-axis and Y-axis respectively, and the fluorescence intensities are indicated by the color scale presenting from blue to red color (Fig. 2).

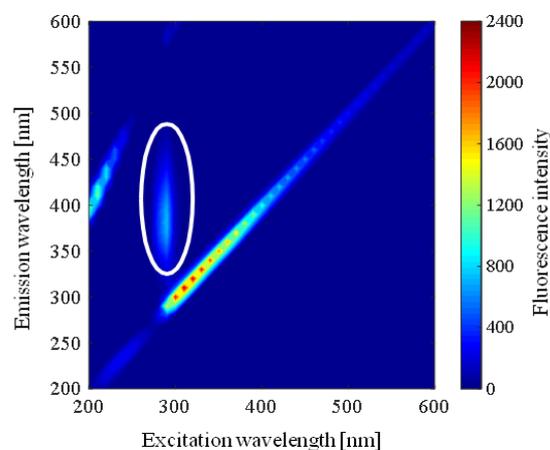


Fig.2. FF contour plot with intensity peak (white circle indicating the peak for ATP)

The FF data contained scattered lights which were problematic when deducing the highest peak intensity from the spectrum¹⁰. Thus, the scattered lights and non-fluorescence data were removed from the original figures when drawing contour plots by using Matlab. 2016a. (The Mathworks Inc., Natick, MA, USA) and the highest peak intensities were used for data analysis.

3. Results and Discussion

3.1 Fluorescence fingerprint of ATP solutions

Figure 3 shows the FF contour maps of non-frozen ATP standard solutions. The color scale, which presents from blue to red, shows the intensities from 0 to 150. However, the values more than 150 are the same color, red. The FFs of the solutions (concentrations 0, 1, 5 and 10 $\mu\text{mol/mL}$) were expressed differently at different pH levels. For the ATP solutions, the FF contour plots showed the variety of configuration among the concentrations and different pH values. The maximum fluorescence intensity was observed at 290 nm and 380 nm (λ_{ex} and λ_{em} , respectively) when the concentration was 10 $\mu\text{mol/mL}$ at pH 5.0. Similar FF contour maps with weaker signals were yielded at lower concentrations of 1 and 5 $\mu\text{mol/mL}$. Moreover, the images of all concentrations were more or less similar at a given pH. However, the FF landscapes changed drastically within a specific concentration when the pH was increased from 5.0 to 8.0 and the peak condition was shifted above pH 6.0.

3.2 Fluorescence fingerprint of frozen ATP samples

In the case of frozen ATP samples, the FFs also showed the different configuration depending on different concentrations and pH values (Fig. 4). The highest peak of fluorescence intensity was obtained at the highest concentration (10 $\mu\text{mol/mL}$) but the peaks of frozen samples were slightly different than that of solutions. This might result from the instrumental condition such as the use of fiber probe, the physical state of the sample, and temperature. Gamal et al. used several wavelength conditions for quantification of ATP-related compounds¹ whereas in the present study only fluorescence peak intensity from a specific wavelength combination has been used (Table 1). Moreover, fluorescence intensities do not behave linearly with pH or concentration in one-wavelength condition and we have to use a multi-wavelength measurement for quantification in a relatively complicated matrix, such as food⁶. The wavelengths of excitation and emission maxima were different for different concentrations to obtain fluorescence peak. However, the trend of intensity

peaks was not so clear among the pH values though the highest peak was observed at 290 nm and 380 nm (λ_{ex} and λ_{em} , respectively) as like as ATP solution.

3.3 Effects of pH

From the FF contour plots of ATP solutions, it was revealed that the strength of the fluorescence signal was influenced by not only the concentration of ATP but also by the pH of samples. Figure 5 shows the relationship between the ATP concentration in non-frozen solutions and fluorescence intensity at different pH values. The highest intensities were observed at pH 5.0 for each concentration, and the intensities declined drastically with increasing pH. At pH 5.0 - 6.0, the fluorescence intensities were highly affected by pH, whereas at pH 7.0 and 8.0 the intensities were not so different. Moreover, the highest intensity peaks were observed at a specific wavelength of excitation and emission (290 nm and 380 nm, respectively) at pH 5.0 - 6.0 for each concentration (Table 1). At pH 7.0 - 8.0, the excitation and emission wavelength shifted to 300 nm and 410 nm, respectively, for all concentrations of ATP solutions, and lower intensities were observed at these pH values.

In the frozen ATP samples, the fluorescence intensity was also influenced by changing concentration and pH. A similar trend of wavelength combination for fluorescence intensity peak was observed though the signals were not as strong as ATP solution. The highest intensity was observed in the concentration of 10 $\mu\text{mol/mL}$. The wavelength conditions (excitation/emission) for 1 and 5 $\mu\text{mol/mL}$ were more or less similar (280 nm / 360-370 nm) at all pH levels. However, at the highest concentration (10 $\mu\text{mol/mL}$) of frozen ATP samples, the fluorescence intensity peaks slightly shifted at a longer excitation and emission wavelength of 290 nm and 370-380 nm, respectively (Table 1).

From the above results, it is revealed that the pH has a potential effect on the FF of ATP solution. There are some reports on the effects of pH on the fluorescence emission of some indole compounds^{11, 12}, whereas no reports on EEM of different food constituents. The results of the present study are supported by the observation that most aromatic molecules are fluorescent in neutral or acidic media, but the presence of a base leads to the formation of non-fluorescent compounds⁸. Serotonin shows a shift in fluorescence emission from neutral pH to strong acidic pH¹¹. The fluorescence of amino substituted proteins is notably influenced by pH and the possible reasons beyond that the intramolecular charge migration as well as a change of molecular structure of tryptophan analogs¹² which supports our study.

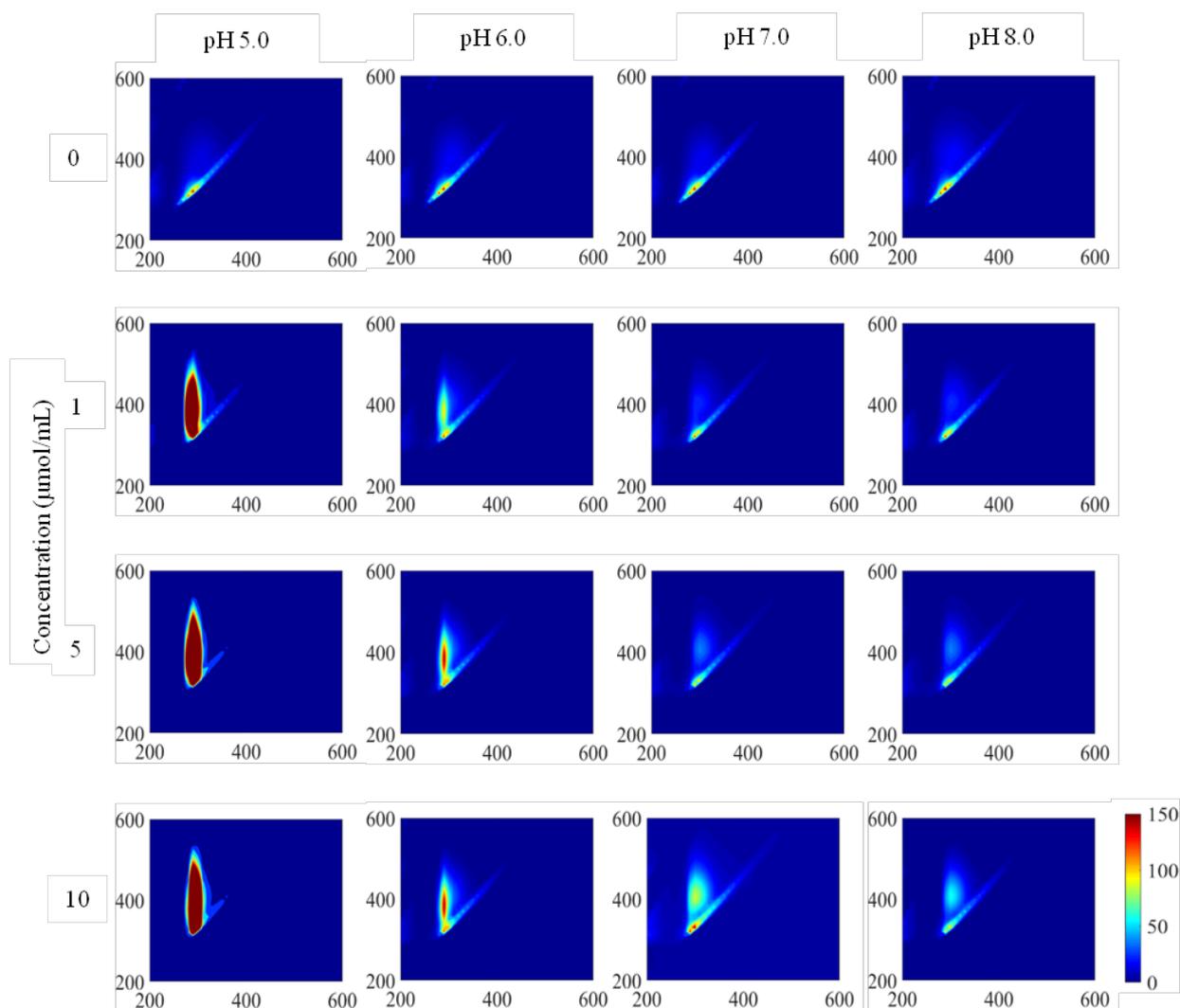


Fig.3. FF contour plots of non-frozen ATP solutions (X-axis = λ_{ex} and Y-axis = λ_{em} in nm)

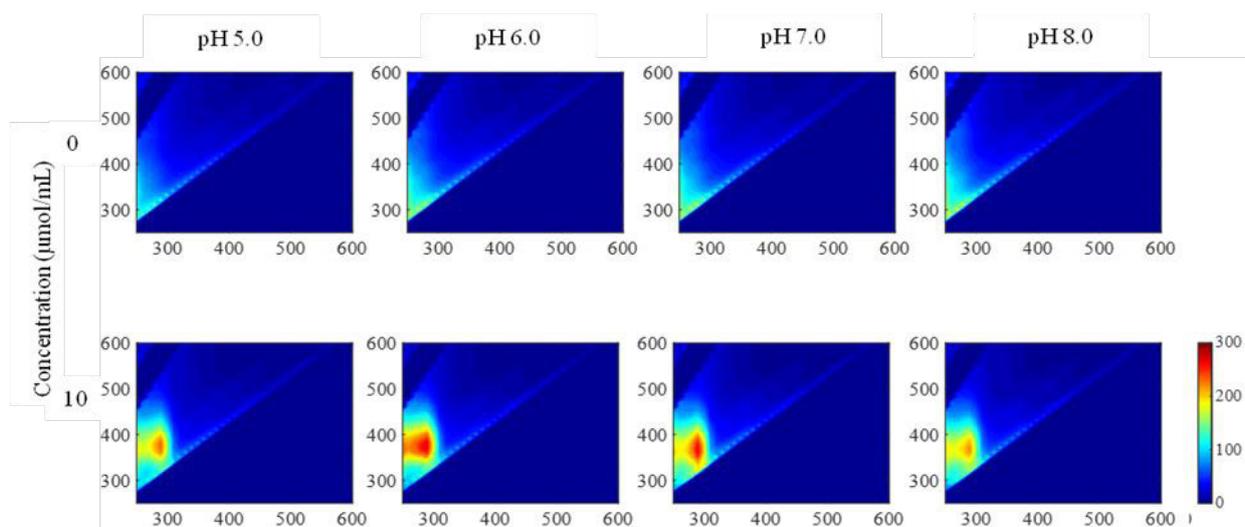


Fig.4. Representative FF contour plots of frozen ATP samples (X-axis = λ_{ex} and Y-axis = λ_{em} in nm)

However, in the frozen samples, the effects are not so prominent and the FF was simultaneously influenced by concentration, pH and temperature. Although a system with multiple constituents ideally yields an FF representing the sum of the fluorescence contribution from each of the inherent fluorophores has been established⁴⁾, the influence of pH and temperature on the FF of ATP was obscure. Therefore, the FF of the frozen sample may be affected by the interaction among the factors as observed in this study.

Table 1. Different wavelength combinations in where highest fluorescence intensity peaks were observed

ATP (μmol/mL)	pH	Peak condition of ATP solution (Excitation / Emission) nm	Peak condition of frozen ATP samples (Excitation / Emission) nm
1	5.0	290/380	280/360
	5.5	290/380	280/360
	6.0	290/380	280/360
	7.0	290/390	280/360
	8.0	300/400	280/360
5	5.0	290/380	280/370
	5.5	290/380	280/370
	6.0	290/380	280/370
	7.0	300/400	280/370
	8.0	300/410	280/370
10	5.0	290/380	290/370
	5.5	290/380	290/370
	6.0	290/380	290/380
	7.0	300/410	290/370
	8.0	300/410	290/370

Finally, it can be concluded that pH exerts a large effect on the FF data of ATP standard solutions. Moreover, the fluorescence intensities of the frozen samples are slightly affected by pH. Thus, it will be needed to quantify the ATP content in fish meat at both raw and frozen states to verify the effects of pH on the FF for the practical application in the food processing industry. Additionally, the effects of pH not only on ATP but also all ATP-related compounds need to be investigated to specify the freshness condition of fish. Therefore, further study will be needed to clarify the mechanism of pH effects on multi-constituent food materials as well as to improve the accuracy of the method by using multivariate analysis.

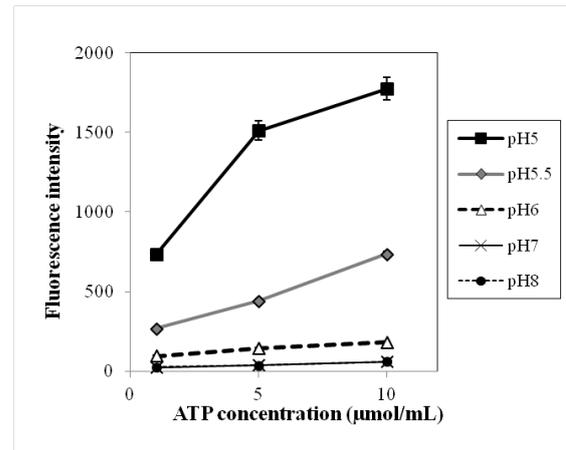


Fig.5. Fluorescence intensities of ATP solutions at highest peak conditions

4. Conclusion

The FF data of both non-frozen and frozen ATP were influenced by pH. Thus, the present study will be valuable since it clearly shows the importance of pH and temperature of the sample when observing ATP in food by using fluorescence.

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