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Analytical Methods

Quantitative measurement of metmyoglobin in tuna flesh via electron paramagnetic resonance

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ABSTRACT

The potential of electron paramagnetic resonance (EPR) spectroscopy in the quantitative determination of metmyoglobin (metMb) in tuna flesh was examined and compared with conventional visible spectrophotometry (VIS). Both fresh and stored tuna samples were directly subjected to EPR measurement at −150 °C without pigment extraction, and their metMb concentrations ($[metMb_{TUNA}]_{EPR}$) were determined from a calibration curve. A linear calibration curve with good correlation ($R^2 = 0.987$) was obtained by a plot between EPR intensities and the known $[metMb_{CALI}]_{VIS}$ concentrations, where $[metMb_{CALI}]_{VIS}$ is the concentration of metMb obtained from visible spectrophotometry for the stock metMb solution. The results show that differences between $[metMb_{TUNA}]_{EPR}$ and $[metMb_{TUNA}]_{VIS}$ for tuna meats are negligible at low concentrations of metMb. However, $[metMb_{TUNA}]_{EPR}$ tends to be higher than $[metMb_{TUNA}]_{VIS}$ at a higher concentration of metMb. This is probably due to incomplete pigment extraction from tuna samples that have been stored for a long period of time. This results in experimental inaccuracy of $[metMb_{TUNA}]_{VIS}$ at higher concentrations of metMb. The overall results suggest that the EPR method is a suitable technique for quantitative measurement of metMb in tuna meat without pigment extraction. Since the EPR method operates at −150 °C, this technique could also be very useful in determining the metMb content in frozen tuna meat during low-temperature storage without thawing.

1. Introduction

The colour of tuna meat is an extremely important characteristic influencing the consumer's purchase decision, especially when it is directly served as sashimi (thinly sliced raw seafood). The red colour of meat depends upon the concentration of myoglobin (Mb) and its derivatives (Faustman, Yin, & Nadeau, 1992; Hood, 1980). During preservation, desirably red tuna meat undergoes discolouration and develops an undesirable brown colour, which results from oxidation of ferrous Mb (deoxymyoglobin, deoMb and oxymyoglobin, oxyMb) derivatives to ferric metmyoglobin (metMb) (Bito, 1965; Bito, 1976). A variety of methodologies of colour measurement has been proposed to observe this discolouration phenomenon in meat extracts; for example, visible spectrophotometry (Bito, 1965; Broumand, Ball, & Stier, 1958; Krzywicki, 1982; Tang, Faustman, & Hoagland, 2004), meat surface via reflectance spectrophotometry (Krzywicki, 1979; Stewart, Zipser, & Watts, 1965) or colorimetry (Ochiai, Chow, & Watabe, 1988).

Both the reflectance spectrophotometry and colorimetry methods are rapid and can reflect colour as well as observation by consumers can (Faustman & Phillips, 2001). However, they are not quantitative determination methods. However, visible spectrophotometry is widely employed to assess meat colour in terms of total Mb concentration and the relative proportions of its derivatives; however, pigment extraction prior to spectrophotometric measurement is necessary. The extraction procedure is cumbersome and destroys the sample (Govindarajan, 1973; Stewart et al., 1965). Additionally, the extraction process may cause production of Mb derivatives not originally present in the meat, for example, by extraction in the presence of oxygen (Dean & Ball, 1960; Stewart et al., 1965), improper filtration (Ehira, Uchiyama, & Kakuda, 1984), using water or high pH buffer as a solvent for extraction (Krzywicki, 1982; Warriss, 1979), and even extraction at high temperature.

Currently, there is a soaring demand for fresh and frozen tuna in the Japanese market and worldwide. The tuna industry has recognised the importance of colour stability to tuna meat marketability. Consequently, tuna is customarily kept in the frozen state immediately after harvest until transportation to local markets to prolong the colour shelf-life of the meat (Chow, Ochiai, & Watabe, 2004; Sasayama, 1984; Zhao, Kolbe, & Craven, 1998). To assess the colour change of fish meat during frozen preservation, thawing is commonly required prior to colour measurements. However, there is a report that the discolouration of red muscles in frozen skipjack and tuna occurs easily during and after thawing (Miki & Nishimoto,

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1987). Therefore, until recently, there has not been a concrete method of colour measurement for frozen tuna completely free from this limitation. Hence, methodologies without pigment extraction and thawing are required to precisely and accurately evaluate the colour change in fresh and frozen fish meat. The precise and accurate evaluation of colour change in fish meat will improve understanding of its mechanism.

Electron paramagnetic resonance (EPR) spectroscopy is one of the useful and powerful spectroscopic methods based on absorption of microwaves by a population of molecules possessing unpaired electrons in the presence of an external magnetic field. Using this method, EPR can easily and independently detect paramagnetic species in the raw material and their environment. EPR has long been used for intensive studies of paramagnetic metalloproteins including metMb (Iizuka & Yonetani, 1970). Typically, EPR studies measure the spin state of the iron atom in haemoproteins. The position of the EPR signal can provide specific information on the iron atom in haem, such as the nature of ligand bindings, symmetry, orientation, and spin state, including the haem environment (Gibson, Ingram, & Schonland, 1958; Ingram & Bennett, 1955). Although other metalloproteins, which are chemical combinations of protein atoms with ions of other metals such as zinc (e.g. DNA polymerase), magnesium (e.g. glucose 6-phosphatase), cupric (e.g. cytochrome oxidase), may exist in ordinary tuna meat, it is found that electron configurations for most of these metal ions differ from that of ferric iron in metMb. The difference in electron configurations results in the difference of the pattern of EPR spectra including the corresponding g values i.e., locations of EPR signals. Therefore, there is no interference from other metalloproteins in the observed EPR signals of ferric iron in metMb. However, the EPR signals of metMb for tissue might be interfered from methaemoglobin (metHb), which gives the similar EPR signals to metMb. Since normally haemoglobin is rather lost easily during handling and storage, the primary pigment retained by the intracellular structure is Mb (Livingston & Brown, 1981). As a result, the effect of metHb on the EPR signals of metMb might be negligible. Therefore, the individual information on metMb in tuna meat, which is a heterogeneous complex system, should be efficiently identified from the analysis of the EPR signal. Moreover, such EPR studies in metMb have not required light transmission of the sample and must operate at very low temperatures because the spectra are difficult to analyse at ambient temperature (Iizuka & Yonetani, 1970). These characteristics are interesting for the tuna industry in that frozen fish meat can be directly subjected to EPR measurement without pigment extraction and thawing. EPR measurement is potentially useful for quantitative determination of metMb formation in fish meat during preservation, especially frozen storage.

In this study, we have attempted to examine the potential of the EPR method as a novel method to determine metMb concentration in tuna meat. In addition, we compared metMb concentrations determined by the EPR method and those determined by visible spectrophotometry, which has been employed conventionally.

2. Materials and methods

2.1. Metmyoglobin solution preparation for calibration curve

Equine skeletal Mb (Sigma–Aldrich Co., Tokyo, Japan) was dissolved in 40 mM sodium phosphate buffer (pH 6.8) at 10 mg/ml. To ensure that all Mb compounds were completely converted to Fe^{3+}, 1 mg of potassium ferricyanide (Wako Pure Chemical Industries, Ltd., Osaka, Japan) was added to 1 ml of the prepared stock myoglobin solution. The excess ferricyanide was removed by dialysis against the same buffer. All procedures were carried out at 4 °C.

The stock metMb solution was diluted into several concentrations. The concentration of an aliquot of each dilution was determined according to the method of Warriss (1979) with some modifications. A few micrograms of sodium cyanide (Wako Pure Chemical Industries, Ltd.) were added to each aliquot to convert the pigments to the cyanmet forms, and the aliquot was then centrifuged at 15,000 rpm for 1 h at 4 °C, using a RS-18GL centrifuge (Tomy Seiko Co., Ltd, Tokyo, Japan). The supernatant was subjected to absorbance measurement at 540 nm using a V-630BIO UV–VIS spectrophotometer (Jasco, Tokyo, Japan) and 40 mM phosphate buffer, pH 6.8, as a blank. The metMb concentrations were calculated from the absorbance at 540 nm (A_{540}) using an extinction coefficient of 11.3 mM^{-1} cm^{-1} (Drabkin, 1947) as follows:

$$\text{Metmyoglobin(mM)} = A_{540}/(11.3\ \text{mM}^{-1}\ \text{cm}^{-1} \times 1\ \text{cm}) \quad (1)$$

2.2. Fish sample preparation

Fresh bluefin (*Thunnus thynnus*) and bigeye (*T. obesus*) tuna were purchased as raw fillets from a local fish retailer. For the bluefin tuna sample, the tuna fillet was obtained from a single portion of ordinary dorsal muscle. For the bigeye tuna, the samples were taken from various tuna fillets of ordinary dorsal muscle. Tuna fillets were cut into pieces having dimensions of 0.5 × 4 × 6 cm. All samples were wrapped with film made from polyethylene and polypropylene (Mitsui Chemicals Fabro Inc., Tokyo, Japan), and packed individually in zip-lock packs (Asahi Kasei Home Products Corporation, Tokyo, Japan) prior to storage at 5 °C (±1 °C) for approximately 1 week. Fish fillet was randomly chosen for analysis of metMb content with both spectrophotometry and EPR methods at various times. For EPR measurement, a small amount of tuna meat was randomly taken by piercing a plastic cylinder pipe (approximately 3 mm inside diameter) in the fish fillet. The fish sample was then pulled out from the pipe and frozen immediately with liquid nitrogen. This sampling method was done throughout the fish fillet, and the fish samples were kept in a freezer at −90 °C until the determination with EPR method. The residue of fish fillet was minced in a cold mortar (4 °C), and then immediately employed to determine the metMb content with spectrophotometry method.

2.3. Determination of metmyoglobin concentration in fish meat samples with the visible spectrophotometry method

Pigments in fish meat samples were extracted according to the method of Lee, Hendricks, and Cornforth (1999) with some modifications. The minced sample (2 g) was placed into a 50-ml polypropylene centrifuge tube, and 20 ml ice-cold phosphate buffer (pH 6.8, 40 mM) was added. The mixture was homogenised for 10 sec at 10,000 rpm with ART-MICCRA D-8 (ART Moderne Labortechnik, Hugelheim, Germany). The homogenised sample was centrifuged at 8000 rpm for 30 min at 4 °C, using a RS-18GL centrifuge (Tomy Seiko Co., Ltd.). In order to avoid any turbidity of the extracts, the supernatant was filtered with 0.3-μm filter paper (Nihon Milipore Kogyo K.K., Yonezawa, Japan).

Half of the supernatant was subjected to measurement of metMb percentage. The absorption spectra of myoglobin derivatives were determined using a V-630BIO UV–VIS spectrophotometer (Jasco). The spectra were recorded from 350 to 750 nm at the scanning rate of 1000 nm/min using 40 mM phosphate buffer, pH 6.8 as a blank. The percentage of metmyoglobin (%metMb) was calculated with the following equation (Tang et al., 2004):

$$\%\text{metMb} = -0.159R_1 - 0.085R_2 + 1.262R_3 - 0.52 \quad (2)$$

where $R_1 = A_{582}/A_{525}$, $R_2 = A_{557}/A_{525}$, $R_3 = A_{503}/A_{525}$.

The other half of the supernatant was subjected to measurement of total Mb concentration according to method of Warriss (1979). A few micrograms of potassium ferricyanide and sodium cyanide were added to the supernatant to convert the pigments to the cyanmet forms, and the sample was then centrifuged at 15,000 rpm for 1 h at 4 °C, using a RS-18GL centrifuge (Tomy Seiko Co., Ltd.). The supernatant was subjected to absorbance measurement at 540 nm using a V-630BIO UV–VIS spectrophotometer (Jasco) and 40 mM phosphate buffer, pH 6.8, as a blank. The total myoglobin concentrations were calculated from Eq. (1). The metMb concentration (μmol/g sample) was calculated from %metMb and total myoglobin concentrations. The measurements were performed in triplicate.

2.4. Determination of metmyoglobin concentration in fish meat sample with the EPR method

2.4.1. EPR sampling and measurement

For measurement of metMb solution, 0.4 cm^3 of the stock metMb solution of each concentration was placed into a quartz EPR sample tube (5 mm of the outside diameter), and then frozen slowly with liquid nitrogen in order to prevent fracture of EPR tube. The EPR tube containing a frozen solution was kept in liquid nitrogen prior to EPR measurement.

For determination of metMb concentration in a meat sample, frozen pierced meat samples were taken from freezer and then kept immediately in liquid nitrogen. Among these samples, few of them were randomly selected and packed into an EPR tube until they reached 3.7 ± 0.1 cm in height (corresponding to 0.4 cm^3) and kept in liquid nitrogen prior to EPR measurement. The actual sample weight in each EPR tube was recorded and used for determination of the metMb concentration per gram of meat sample.

EPR spectra were recorded at −150 °C with an EPR spectrometer, JES-TE300 (JEOL Co., Tokyo, Japan) operating at 9.11 ± 0.1 GHz (X band). Typical acquisition parameters are as follows: microwave power, 1 mW; modulation width, 1 mT; spectral range, 60–360 mT; sweep time, 4 min; time constant, 0.1 sec; number of data points, 4096; and number of scans, 1. The standard metMb solutions in the concentration range of 0.011–0.094 mM were measured with amplitude = 630, whilst those of 0.081–0.228 mM were measured with amplitude = 160. The Mn^{2+} marker (JEOL Co., Tokyo, Japan) was used as a reference for the magnetic field and the peak intensity. EPR spectra for the sample and the Mn^{2+} marker were simultaneously recorded. The measurements were performed in triplicate.

2.4.2. EPR spectra analysis

Since the spectra for the Mn^{2+} marker have a typical Gaussian line shape, the intensity of the Mn^{2+} reference (I_R) is proportional to $w_R{}^2 h_R$, where w_R is the half-width and h_R is the height of the line for the Mn^{2+} marker signal, respectively (Weil, Bolton, & Wertz, 1994). However, the spectra for metMb typically show an anisotropic line shape, with $g_\perp$ (ca. 6) and $g_\parallel$ (ca. 2) (Svistunenko, Sharpe, Nicholls, Wilson, & Cooper, 2000). The exact g value of the unpaired electron in a molecule depends on the structure of that molecule (Macomber, 1988). In this study, the negative peak at around 325 mT (g = ca. 2) was very weak and difficult to use for quantification of the intensity of the sample (I_S). The anisotropic spectrum is assumed to be the superposition of Gaussian lines; therefore, I_S would be proportional to $w_S{}^2 h_S$, where w_S and h_S are determined from the positive and negative peak at around 100 mT (g = ca. 6), as shown in Fig. 1F. As a result, the ratio of I_S/I_R is expressed as follows:

$$I_S/I_R = w_S^2 h_S / w_R^2 h_R \quad (3)$$

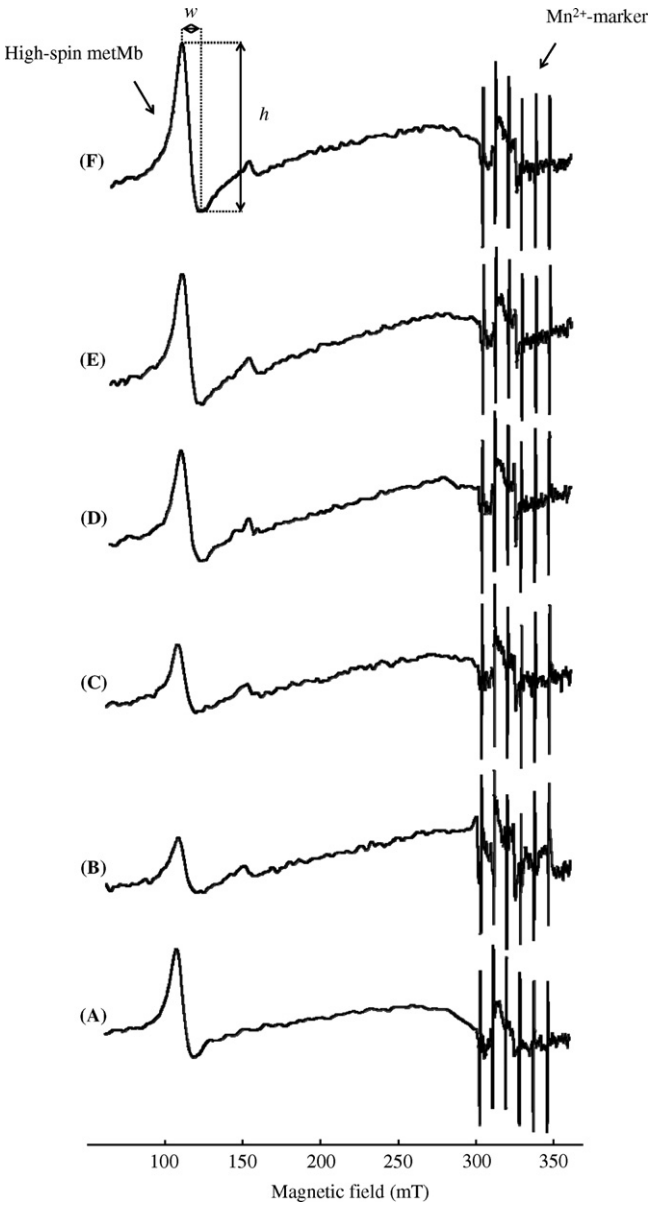

Fig. 1. EPR spectra of metMb for 0.09 mM of equine metMb solution (A), fresh bluefin tuna meat (B), and bluefin tuna meat stored at 5 °C for different storage times. (C), (D), (E), and (F) represent data for bluefin tuna stored at 5 °C for 1, 3, 4, and 5 days, respectively. w and h represent the half-width and the height of the line for EPR signal of metMb, respectively. EPR signals in the range of 100–150 mT represent high-spin haem with g = 5.80 for metMb solution (A) and g = 5.78 for tuna meat samples (B–F). EPR signals in the range of 300–350 mT represent Mn^{2+} marker signals. EPR measurements were carried out with amplitude = 630 at −150 °C.

The ratio of I_S/I_R was used as a dimensionless parameter to prepare the calibration curve and thus to estimate the metMb concentration in fish meat.

3. Results and discussion

3.1. Quantitative analysis of metmyoglobin by EPR method

3.1.1. Calibration curve

EPR spectra from the stock metMb solution are shown in Fig. 1A. A large signal in the range of 100–150 mT represents the high-spin haem signal with g = 5.80. Svistunenko et al. (2000) reported that the high-spin haem in metMb, where the sixth ligand

of its ferric iron atom bonds to a water molecule, provides the large EPR signal at $g = 5.85$ (zero-line crossing) and the tiny signal at $g = 2$. The high-spin signal with $g = 2$ (at around 325 mT) in our results was very weak and was not recognised. Since the ferric iron atom in the metMb molecule, which is a product of Mb autoxidation in meat, is always bound to a water molecule (Livingston & Brown, 1981), the high-spin haem EPR signal in the range of 100–150 mT was employed to estimate the I_S value corresponding to the metMb species. However, the six signals in the range of 300–350 mT represent Mn^{2+} marker (Fig. 1A). The average value of I_R was determined from these six Mn^{2+} signals.

Since deoxyMb has no unpaired electron i.e., is diamagnetic, and oxyMb has an even number of unpaired electrons (Kotani, 1968), both deoxyMb and oxyMb could not be detected with EPR. Thus, only the absolute metMb concentration is determined with EPR.

The calibration curve for the determination of the metMb concentration was constructed as shown in Fig. 2. The results showed that there is a linear relationship between the ratio I_S/I_R and the concentration of metMb obtained from visible spectrophotometry for the stock metMb solution ($[\text{metMb}_{\text{CALI}}]_{\text{VIS}}$) in the range 0.011–0.228 mM. It should be noted that this concentration range covers the total Mb concentration typically retained in ordinary muscle of yellowfin and bluefin tuna (Brown, 1961; Chow, Ochiai, Watabe, & Hashimoto, 1988). The linear regression equation was:

$$I_S/I_R = k[\text{metMb}_{\text{CALI}}]_{\text{VIS}} \quad (4)$$

with a good correlation coefficient ($R^2 = 0.987$). k is a constant for our equipment and the measurement conditions. Therefore, the calibration curve will vary in different EPR machines. The calibration curve shown in Fig. 2 was employed to determine the metMb concentration in tuna meat.

3.1.2. Quantification of metmyoglobin in tuna meat

EPR spectra of metMb for fresh and stored bluefin tuna meats at 5 °C for different storage times are shown in Fig. 1B–F. The tuna meat samples gave the EPR signal of the high-spin form of haem bonding to water at $g = 5.78$ (zero-line crossing), similar to the EPR spectra of the standard metMb solution shown in Fig. 1A. The distinct EPR signal at $g =$ ca. 5.8, which was observed for both tuna meat sample and the metMb solution (Fig. 1), suggested that it was not substantially interfered from other metalloproteins. For tuna meat samples, it is obvious that the high-spin haem signal at $g = 5.78$ became larger with increasing storage period (Fig. 1B–F).

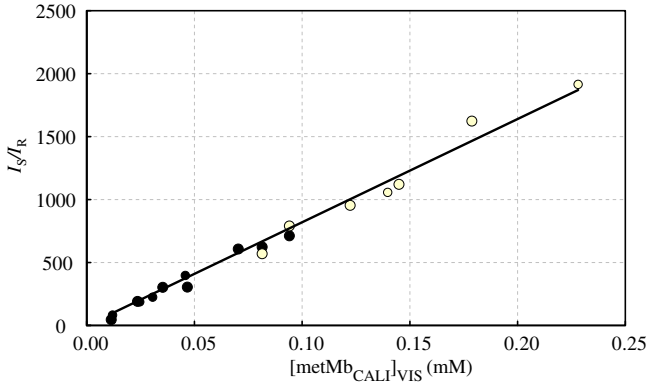

Fig. 2. Calibration curve of the relationship between the ratio I_S/I_R obtained from EPR and the metMb concentration obtained from visible spectrophotometry for the stock metMb solution ($[\text{metMb}_{\text{CALI}}]_{\text{VIS}}$). Closed and open circles represent data measured with amplitude = 630 and 160, respectively. The linear regression equation can be written as $I_S/I_R = 8198.2\ [\text{metMb}_{\text{CALI}}]_{\text{VIS}}$ with correlation coefficient $R^2 = 0.987$.

The combination of these results and the good linear correlation for the calibration curve indicated that the EPR measurement would be suitable for determination of the metMb concentration in fish meat.

The I_S value for the tuna meat sample was calculated from the high-spin haem EPR signal in the range of 100–150 mT, and the I_R value was determined from the average value of the six Mn^{2+} signals in the range of 300–350 mT. Using the ratio I_S/I_R, the concentration of metMb for tuna samples ($[\text{metMb}_{\text{TUNA}}]_{\text{EPR}}$) was determined from the calibration curve (Eq. (4), Fig. 2). Sample weight and the corresponding 0.4 cm^3 of the stock metMb solution were used to express the concentration of metMb in tuna meat in micromoles per gram of sample.

The metMb concentrations of tuna meat determined by the EPR method are shown in Table 1. The results reveal that the errors in triplicate measurement are in the range of ± 0.018 μmol/g sample. EPR measurement uses a small amount of sample. This means that the concentration of metMb obtained from the EPR method can be easily affected by the non-uniform distribution of metMb in the fish sample. This probably caused the large errors in the triplicate measurement (Table 1). These results suggest that the EPR method can be used as a non-destructive method for determination of the metMb concentration in fish meat.

3.2. Quantitative analysis of metmyoglobin by visible spectrophotometry

Examples of the optical absorption spectra of tuna extracts for fresh bluefin tuna and bluefin tuna stored at 5 °C for 7 days are shown in Fig. 3. It is obvious that the absorption maxima at 503 and 632 nm increased with storage time, as shown by an arrow in Fig. 3. These two bands represented the spectral characteristics of ferric metMb in the high-spin state (Iizuka & Yonetani, 1970). The absorption at 503 nm was used to estimate the %metMb (Tang et al., 2004). In other words, the %metMb measured from conventional spectrophotometry was determined from the absorption maxima of high-spin derivatives of haem in metMb.

The absolute metMb concentration ($[\text{metMb}_{\text{TUNA}}]_{\text{VIS}}$) was calculated from the total Mb concentration and the %metMb. The total Mb concentrations and the %metMb and the absolute metMb concentrations determined from visible spectrophotometry are shown in Table 1. The results revealed that the errors in the triplicate measurement were in the range of ± 0.005 μmol/g sample. For bluefin tuna, the tuna fillet was obtained from a single portion of ordinary dorsal muscle. Hence, the results from different storage times could be compared. The results for fresh bluefin tuna and bluefin tuna stored at 5 °C for different periods of time are shown in samples 1 and 5–9 (Table 1), respectively. It is seen that there was a decrease in the total Mb concentration when storage time was prolonged for bluefin tuna, especially after 4 days storage. The result is in accordance with Chaijan, Benjakul, Visessanguan, and Faustman (2005), who reported that total extractable pigment content in both dark and ordinary muscle from sardine and mackerel gradually decreased as the storage time increased during iced storage. In addition, Chen, Chow, and Ochiai (1996) reported that the Mb extracting efficiency decreased in washed milkfish during iced or frozen storage. Chaijan et al. (2005) reported that the decrease in pigment extractability was due to pigment possibly undergoing oxidation or denaturation during storage, leading to the higher pigment content remaining or bound in the muscle. This might be associated with the cross-linking between protein, such as Mb, and the lipid oxidation products. These results suggest that visible spectrophotometry can satisfactorily evaluate the autoxidation of tuna Mb only in the initial period of storage.

Table 1
Metmyoglobin concentration determined by EPR and visible spectrophotometry methods ($n = 3$)

	Visible spectrophotometry			EPR	Material
	Total pigment (μmol/g sample)	%metMb	[metMb] (μmol/g sample)	[metMb] (μmol/g sample)	
1	0.258 ± 0.003	18.561 ± 0.349	0.048 ± 0.000	0.045 ± 0.004	Fresh bluefin tuna
2	0.181 ± 0.003	11.578 ± 0.561	0.021 ± 0.002	0.024 ± 0.004	Fresh bigeye tuna
3	0.194 ± 0.005	14.094 ± 0.348	0.027 ± 0.003	0.029 ± 0.005	Fresh bigeye tuna
4	0.162 ± 0.005	10.546 ± 0.385	0.017 ± 0.000	0.012 ± 0.006	Fresh bigeye tuna
5	0.250 ± 0.003	21.173 ± 0.464	0.053 ± 0.002	0.070 ± 0.013	Stored bluefin tuna, 1 day at 5 °C
6	0.254 ± 0.013	35.547 ± 0.221	0.090 ± 0.002	0.115 ± 0.002	Stored bluefin tuna, 3 days at 5 °C
7	0.247 ± 0.004	41.283 ± 0.230	0.102 ± 0.002	0.102 ± 0.018	Stored bluefin tuna, 4 days at 5 °C
8	0.232 ± 0.003	51.913 ± 2.460	0.120 ± 0.005	0.133 ± 0.006	Stored bluefin tuna, 5 days at 5 °C
9	0.242 ± 0.005	79.525 ± 0.268	0.192 ± 0.005	0.239 ± 0.012	Stored bluefin tuna, 7 days at 5 °C
10	0.127 ± 0.003	26.442 ± 0.393	0.034 ± 0.000	0.026 ± 0.001	Stored bigeye tuna, 1 day at 5 °C
11	0.119 ± 0.006	37.629 ± 0.367	0.045 ± 0.003	0.047 ± 0.003	Stored bigeye tuna, 3 days at 5 °C
12	0.120 ± 0.003	39.854 ± 0.831	0.048 ± 0.002	0.052 ± 0.004	Stored bigeye tuna, 4 days at 5 °C
13	0.174 ± 0.001	22.836 ± 0.373	0.040 ± 0.000	0.033 ± 0.009	Stored bigeye tuna, 4 days at 5 °C
14	0.118 ± 0.004	50.987 ± 4.374	0.060 ± 0.003	0.056 ± 0.011	Stored bigeye tuna, 5 days at 5 °C
15	0.173 ± 0.001	25.366 ± 0.157	0.044 ± 0.000	0.050 ± 0.001	Stored bigeye tuna, 5 days at 5 °C
16	0.164 ± 0.008	42.094 ± 0.789	0.069 ± 0.004	0.075 ± 0.012	Stored bigeye tuna, 10 days at 5 °C
17	0.079 ± 0.001	42.041 ± 1.263	0.033 ± 0.001	0.019 ± 0.003	Bigeye tuna, freeze-thawed two times, and stored at 5 °C for 1 day
18	0.079 ± 0.003	51.056 ± 0.198	0.040 ± 0.002	0.029 ± 0.013	Bigeye tuna, freeze-thawed two times, and stored at 5 °C for 2 days

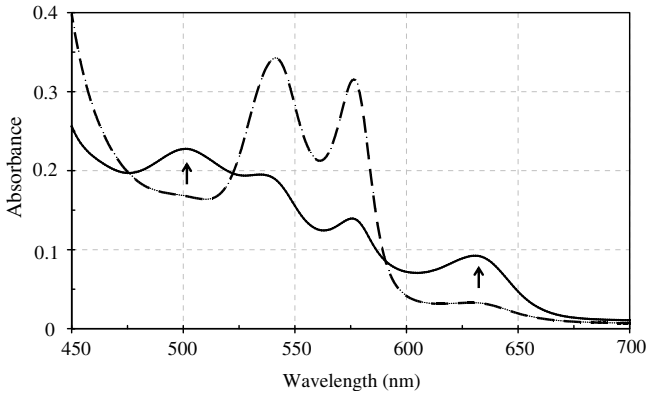

Fig. 3. Optical spectra of bluefin tuna extracts. Dashed and solid lines represent the data for fresh tuna and tuna stored at 5 °C for 7 days, respectively. The arrows show the increase of optical absorption at 503 and 630 nm with increasing storage time.

3.3. Comparison of the results from EPR and visible spectrophotometry methods

The results obtained from the EPR method were presumed to be determined from the signal of high-spin derivatives of haem in metMb. It is known that the line shape of the high-spin EPR signal in the range of 100–150 mT for metMb (Fig. 1) is indistinguishable from that of methaemoglobin (metHb) (Peisach, Blumberg, Wittenberg, & Wittenberg, 1968; Svistunenko, Sharpe, Nicholls, Wilson, & Cooper, 2000). The %metMb measured from conventional spectrophotometry was also determined from the absorption maxima of high-spin derivatives of haem in metMb (Bito, 1965; Tang, Faustman, & Hoagland, 2004) (Fig. 3). Additionally, Mb and Hb have the same optical spectral properties (Govindarajan, 1973). Consequently, the results obtained from both EPR and spectrophotometry methods involve the contributions of the high-spin forms of haem in both metMb and metHb in fish meat. This consideration suggests a similarity in the basis of data analysis for both methods. Therefore, the data obtained using these two methods should be correlated. Nevertheless, there were some differences between them that are demonstrated in the following.

Visible spectrophotometry requires careful pigment extraction for light transmission of the sample; in contrast, the EPR method does not require this, which means that fish meat can be directly subjected to the EPR method. It is known that the difference in the depth of oxygen penetration to meat during storage results in difference in oxygen partial pressure in each layer of the meat. This also results in the difference in the concentration of each Mb derivatives in each layer of the meat during storage. For example, the metMb layer existing in a certain depth in the meat increases and moves toward to the meat surface during storage. To avoid this effect, a whole fish fillet may be minced and homogenised before extraction for visible spectrophotometry method, whilst small amount of tuna samples may be taken from the top to bottom pass through the fish fillet for EPR method. However, the height of fish sample, which can be pack into an EPR tube, is limited; for example, 3.7 cm in this study. Therefore, the results from an improper taking fish samples from the fish fillet which is thicker than the limit of packing fish sample in the EPR tube may be affected by the depth of oxygen penetration in fish fillet. Furthermore, our proposed EPR method operating at −150 °C would be free from the problem of the autoxidation of Mb, which may occur during colour measurement at ambient temperature. These differences in the determining process may lead to differences in the obtained concentrations of metMb between the two methods. Comparisons between the capabilities of the EPR and visible spectrophotometry methods as quantitative methods of measurement of meat pigments are summarised in Table 2.

The relationship between the concentration of metMb determined from the EPR method and visible spectrophotometry for 18 samples taken from various types of tuna flesh and a variety of preservation conditions is shown in Fig. 4. The difference in the results obtained by these two methods was negligible at low concentrations of metMb. However, at high concentrations of metMb, the concentrations of metMb obtained using the EPR method were higher than those obtained using visible spectrophotometry. In this study, the small amounts of tuna samples were taken by piercing the pipe pass through the fish fillet having 0.5 cm in thickness for EPR method. This means that the fish meat from surface to surface was determined with EPR method. The residue of the fish fillet was minced prior to determine with visible spectrophotometry. Therefore, the increasing metMb layer existing in a certain

Table 2
Comparisons between the capabilities of the EPR and visible spectrophotometry methods as quantitative methods of measurement of meat pigments

	EPR	Visible spectrophotometry
Sample	Fish sample can be observed directly	Pigment extraction is required
Spectral properties	EPR signal of high-spin form of metMb at g = ca. 6 is used to determine [metMb] Spectra are contributed from both Mb and Hb	Absorption maxima of high-spin form of metMb are used to determine %metMb Spectra are contributed from both Mb and Hb.
Estimation of [metMb]	[metMb] is determined from calibration curve between EPR intensities and [metMb]	[metMb] = %metMb × [total Mb]
Experimental temperature	−150 °C	4 °C

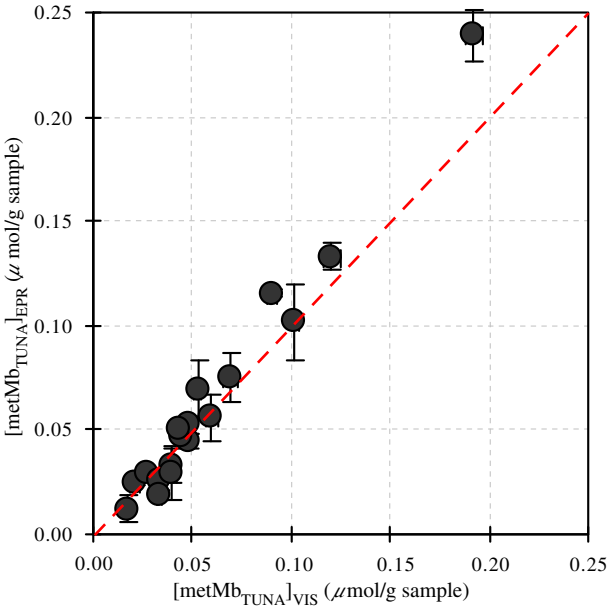

Fig. 4. Comparison of metMb concentration obtained by spectrophotometry ([metMb$_{TUNA}$]$_{VIS}$) and the EPR method ([metMb$_{TUNA}$]$_{EPR}$) for tuna meat samples (n = 3). The dashed line indicates the expected linear relation when the slope is 1.

depth in the meat toward the meat surface during storage has not influenced on the discrepancy in data obtained by EPR and visible spectrophotometry methods. For the results obtained by visible spectrophotometry, as mentioned above, there was a decrease in the total pigment concentration when storage time for bluefin tuna was prolonged, especially after 4 days of storage (Table 1). The cross-linking between protein, such as metMb, and lipid oxidation products, which might result in this decrease in the total pigment concentration, might decrease the mobility of metMb and thus might change EPR spectra. However, EPR spectra were recorded at −150 °C, which was sufficiently low to hinder the mobility of metMb regardless its initial environmental condition; for example, metMb in solution, meat, gel, or cross-linking condition. As a result, the intensity of the observed high-spin haem signal at g = 5.78 would not be affected by the cross-linking of metMb. Therefore, a possible reason for the discrepancy in data obtained by these two methods could be incomplete pigment extraction from stored tuna samples using visible spectrophotometry. These findings suggest that the EPR method is more accurate than visible spectrophotometry for determining the concentration of metMb in stored tuna meat when storage time is prolonged.

4. Conclusion

A novel EPR method for quantitative determination of the concentration of metMb in fish meat was developed, and its feasibility was demonstrated on a limited number of tuna meat samples stored at 5 °C for different storage times. The data obtained by the EPR method were compared with data obtained by conventional visible spectrophotometry. The EPR method does not require pigment extraction from fish meat, and thus the EPR method is more accurate than visible spectrophotometry, particularly for fish meat stored for prolonged periods of time. Since this novel EPR method is operating at a low temperature (−150 °C), it will help in further assessing the autoxidation of Mb in fish meat during frozen storage without thawing. This EPR method can be applied for the determination of the metMb concentration in other red meats such as beef, pork, and chicken.

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