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Analytical Methods

Equations for spectrophotometric determination of relative concentrations of myoglobin derivatives in aqueous tuna meat extracts

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ABSTRACT

The percentage of metmyoglobin (%metMb) in aqueous meat extracts of bigeye and bluefin tuna and beef samples were estimated using previously reported equations derived from the absorption spectra of horse Mb. The results demonstrate that in an aqueous extract, the difference in %metMb estimated by the different equations was negligible for beef samples. Conversely, in an aqueous tuna extract, different %metMb values were obtained with the different equations. The discrepancy in the tuna sample results might be due to differences in absorption spectra for horse and tuna Mb. Therefore, a new set of equations derived from the absorption spectra of bigeye tuna Mb, reported by Matsuura and Hashimoto (1955), was established. The accuracy of the proposed equations was compared with the cyanmetmyoglobin (cyanmetMb) method. The results show that the total Mb concentrations estimated by our proposed equations were in good agreement with the results obtained by the conventional cyanmetMb method (R^2 = 0.984). Therefore, the new set of proposed equations is valid for the spectrophotometric determination of the relative proportions of Mb derivatives and total Mb concentration in aqueous tuna meat extracts.

1. Introduction

The red colour of tuna meat is an important factor used in the evaluation of meat quality, and strongly influences the consumer's purchasing decision. It is known that the red colour of meat depends upon the concentration of myoglobin (Mb) and its derivatives (Faustman, Yin, & Nadeau, 1992; Hood, 1980). During storage, the desired red tuna meat undergoes discolouration and develops an unappealing brown colour, which results from the oxidation of ferrous Mb (deoxymyoglobin, deoMb and oxymyoglobin, oxyMb) derivatives to ferric metmyoglobin (metMb) (Bito, 1965, 1976). With the recent rise in the demand for high-quality fresh and frozen fish in the world market (Catarci, 2004), an increasing amount of research is focusing on the colour changes or Mb oxidation, not only in tuna meat, but also in many other kinds of red fish meats (Benjakul & Bauer, 2001; Chen, 2003; Chaijan, Benjakul, Visessanguan, Lee, & Faustman, 2006; Chaijan, Benjakul, Visessanguan, & Faustman, 2005, 2007; Viriyarattanasak, Matsukawa, Hamada-Sato, Watanabe, & Suzuki, 2008; Viriyarattanasak, Watanabe, & Suzuki, 2007). To assess the oxidation of Mb, many studies have employed visible spectrophotometry and Mb oxidation was commonly reported in terms of the percentage of metMb (%metMb). In visible spectrophotometry, pigment extraction is done prior to absorbance measurement, and then the relative concentrations of Mb derivatives is estimated from the measured absorption spectra using equations derived from the application of Lambert–Beer Law. Almost all of the previously reported equations were derived from absorbance coefficients of horse Mb (Broumand, Ball, & Stier, 1958; Krzywicki, 1979, 1982; Tang, Faustman, & Hoagland, 2004). Since horse and beef Mb are considered to have the same spectrometric profiles (Broumand et al., 1958; Stewart, Hutchins, Zipser, & Watts, 1965; Wolfe, Watts, & Brown, 1978), it is suggested that the relative concentrations of Mb derivatives for aqueous beef extract should be determined by using the equation derived from the absorption spectra of horse Mb. Nevertheless, a suitable equation derived from the absorption spectra of tuna or other fish Mb has not been recently reported.

There are differences in some chemical and physical properties of fish and mammalian Mb, such as the primary structure (Ueki & Ochiai, 2004; Watts, Rice, & Brown, 1980) and absorption spectra (Amano & Tsuyuki, 1975; Brown, Martinez, Johnstone, & Olcott, 1962; Matsuura & Hashimoto, 1955). Matsuura and Hashimoto (1955) reported that for tuna and horse, the absorption spectra of metMb and deoMb in the visible region are similar, but those of oxyMb are quite different. Fig. 1 shows peak of the absorption

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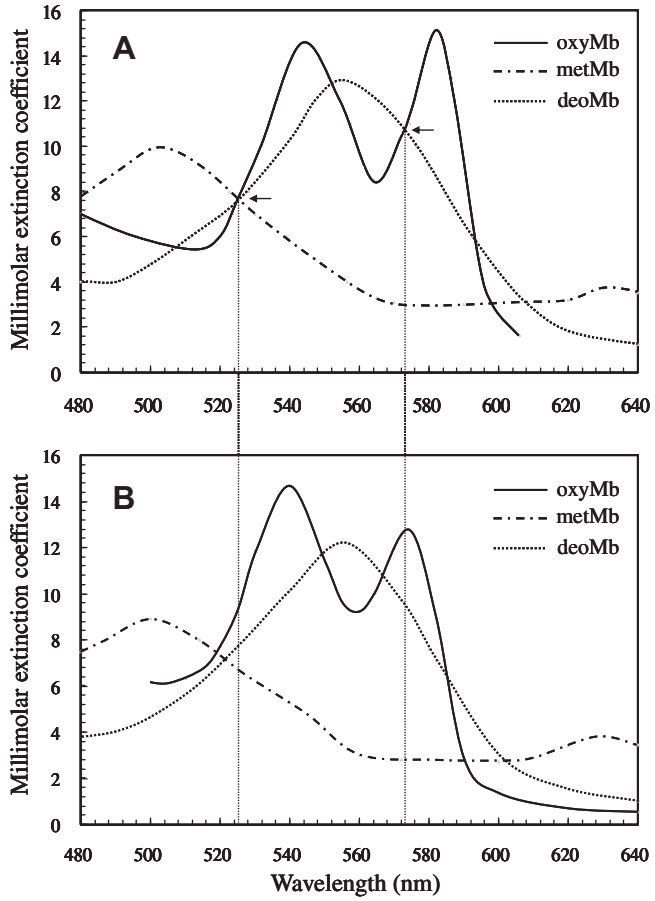

Fig. 1. Absorption spectra of Mb derivatives for (A) horse (Bowen, 1949) and (B) bigeye tuna (Matsuura & Hashimoto, 1955). The arrows show the isobestic point for horse Mb at 525 nm, where the extinction coefficient of three Mb derivatives are equivalent, and at 572 nm, where the oxyMb and deoMb have identical extinction coefficients.

spectra for tuna oxyMb at a shorter wavelength, compared to that of horse oxyMb. Furthermore, the extinction coefficient at the β-maximum (ca. 540 nm) of tuna oxyMb is higher than that at the α-maximum (ca. 580 nm), while a contrasting result is shown for horse oxyMb. Fig. 1 also shows the difference in wavelengths at isobestic points (intersections) between horse and tuna Mb. For example, the oxyMb and deoMb from horse have identical extinction coefficients at 572 nm. However, in tuna this isobestic point is shifted several nanometres to a shorter wavelength. Moreover, in tuna there is no isobestic point at 525 nm, where the extinction coefficients of the three Mb derivatives are equivalent. The isobestic point at 525 nm is characteristically observed in the absorption of horse Mb (Bowen, 1949; Broumand et al., 1958). The wavelengths at the absorption maxima and the isobestic points are commonly selected in the derivation of equations for the estimation of the relative concentrations of Mb derivatives. Therefore, the differences in these parameters between horse and tuna Mb may result in the over- or under-estimation of values when the relative concentrations of tuna Mb derivatives are estimated from equations derived from horse Mb and vice versa (Broumand et al., 1958). Consequently, this may lead to erroneous results and inaccurate interpretations.

Matsuura and Hashimoto (1955) reported that all derivatives of Mb prepared from bigeye and bluefin tuna have almost the same absorption maxima and minima characteristics. Brown et al. (1962) also found that the wavelengths and the absorbances at the absorption maxima of metMb and deoMb from albacore, bluefin, and yellowfin tuna were almost identical. These data suggest that in all Mb derivatives, the absorption spectra from the different species of tuna seem to be identical. Therefore, the absorption spectra data of bigeye tuna Mb, which was reported to be entirely in the visible region for the three Mb redox forms by Matsuura and Hashimoto (1955), was chosen to establish a set of equations for the estimation of the relative concentrations of Mb derivatives in aqueous tuna extracts in the present study.

The objective of this study is to demonstrate the inappropriate determination of the relative concentrations of Mb derivatives in aqueous extracts of tuna samples by using the previously reported equations established from the absorption spectra of horse Mb. Furthermore, a new set of equations derived from the absorption spectra of bigeye tuna Mb, reported by Matsuura and Hashimoto (1955), is proposed. Additionally, we compared total Mb concentrations calculated using a new set of proposed equations and those determined by the cyanmetmyoglobin method (Warriss, 1979).

2. Materials and methods

2.1. Meat sample preparation

Fresh (unfrozen) specimens of bigeye (*Thunnus obesus*) and bluefin tuna (*Thunnus orientalis*), ground beef, and cut beef meat (0.5 × 5 × 8 cm) were purchased from a local retailer. Typically, in Tokyo, fresh fish and beef are transported to a local retailer in the morning, and then they are immediately filleted or ground prior to selling on the same day. Additionally, each tuna specimen used in this study was sold for consumption as a raw fish. It was suggested that fish consumed raw should not contain the %metMb more than 30% (Takai, 2000). The bigeye and bluefin tuna samples were taken from ordinary dorsal muscle. In this study, there were 5 specimens of bigeye tuna, 1 of bluefin tuna, 3 of ground beef, and 1

Table 1
Equations for spectrophotometric determination of the relative concentrations of myoglobin (Mb) derivatives.

	Equations	Reference
(1)	$\%metMb = (1.395-[(A_{572}-A_{700}/(A_{525}-A_{700})]) \times 100$	(Krzywicki 1979; Chen 2003)
(2)	$\%metMb = (1.395-[(A_{572}-A_{730})/(A_{525}-A_{730})]) \times 100$	(Fernandez-Lopez et al., 2003; Krzywicki 1979)
(3)	$\%metMb = (1.395-[(A_{572}-A_{730} \times 1.45)/(A_{525}-A_{730} \times 1.73)]) \times 100$	(Krzywicki 1979; Trout 1990)
(4)	$\%metMb = (-2.514R_1 + 0.777R_2 + 0.800R_3 + 1.098) \times 100$ $\%deoMb = (0.369R_1 + 1.140R_2-0.941R_3 + 0.015) \times 100$ $\%oxyMb = (0.882R_1-1.267R_2 + 0.809R_3-0.361) \times 100$ $R_1 = A_{572}/A_{525}\ R_2 = A_{565}/A_{525}\ R_3 = A_{545}/A_{525}$	(Krzywicki 1982)
(5)	$\%metMb = (-0.159R_1-0.085R_2 + 1.262R_3-0.520) \times 100$ $\%deoMb = (-0.543R_1 + 1.594R_2 + 0.552R_3-1.329) \times 100$ $\%oxyMb = (0.722R_1-1.432R_2-1.659R_3 + 2.599) \times 100$ $R_1 = A_{582}/A_{525}\ R_2 = A_{557}/A_{525}\ R_3 = A_{503}/A_{525}$	(Tang et al., 2004)

of cut beef meat. For the tuna samples, the fillets were cut into $0.5 \times 4 \times 6$ cm pieces. For ground beef samples, patties were formed with a diameter of 8.7 and 0.5 cm thickness. All samples were wrapped with film made from polyethylene and polypropylene (Mitsui Chemicals Fabro, Inc., Tokyo, Japan), and individually packed in zip-lock packs (Asahi Kasei Home Products Corporation, Tokyo, Japan) prior to storage at 0 and 5 °C (±1 °C) for approximately 10 days. Meat samples were randomly chosen for visible spectrophotometric determination at various times.

2.2. Determination of the relative concentrations of Mb derivatives and total Mb concentration

Pigments in meat samples were extracted according to the method of Lee, Hendricks, and Cornforth (1999) with some modifications. The sample (2 g) was first minced in a pre-cooled mortar and then placed into a 50-ml polypropylene centrifuge tube, and 20 ml ice-cold phosphate buffer (pH 6.8, 40 mM, 4 °C) was added. The mixture was homogenised for 10 s at 10,000 rpm with an ART-MICCRA D-8 (ART Moderne Labortechnik, Hugelheim, Germany). The homogenised sample was centrifuged at 7090 *g* for 30 min at 4 °C, using a RS-18GL centrifuge (Tomy Seiko Co., Ltd., Tokyo, Japan). In order to avoid any turbidity of the extracts, the supernatant was filtered with 0.3-μm filter paper (Nihon Millipore Kogyo, Yonezawa, Japan).

Half of the supernatant was directly subjected to measurement of the absorption spectra of Mb derivatives using a V-630BIO UV–vis spectrophotometer (Jasco, Tokyo, Japan). The spectra were recorded from 350 to 750 nm at a scanning rate of 1000 nm/min using 40 mM phosphate buffer (pH 6.8) as a blank. The relative concentrations of Mb derivatives and total Mb concentration were calculated using the equations summarised in Table 1 and our proposed equations.

The other half of the supernatant (approximately 10 ml) was subjected to measurement of total Mb concentration with the cyanmetmyoglobin (cyanmetMb) method (Warriss, 1979). A few micrograms of potassium ferricyanide and sodium cyanide were added to the supernatant to convert the pigments to the cyanmetMb form, and the sample was then centrifuged at 24,910*g* 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 the UV–vis spectrophotometer and 40 mM phosphate buffer (pH 6.8) as a blank. Matsuura and Hashimoto (1955) reported that the extinction coefficient at the absorption maximum (at 540 nm) of the cyanmetMb from bigeye tuna was 10.36 $mM^{-1}\,cm^{-1}$. Therefore, in the present study, the total Mb concentration in aqueous tuna meat extracts was calculated from the absorbance at 540 nm (A_{540}) as follows:

$$\text{Myoglobin}(\text{mM}) = A_{540}/(10.36\ \text{mM}^{-1}\ \text{cm}^{-1} \times 1\ \text{cm})$$

In this study, total Mb concentration was presented in terms of μmol/g sample. The measurements were performed in triplicate.

Table 2
Results of using different equations and cyanmetMb method to determine the relative concentrations of Mb derivatives and total Mb concentration in tuna meat extracts and in solutions of bigeye tuna Mb ($n = 3$).

Material	Storage condition	Relative proportions of Mb redox forms (%)								Total Mb concentration in meat (μmol/g sample)	
		Equation (1)	Equation (5)				Proposed equations				
		metMb	metMb	deoMb	oxyMb	Sum	metMb	deoMb	oxyMb	Proposed equation	CyanmetMb method
Bigeye tuna metMb solution*		97.7	101.8	−6.7	4.6	99.7	99.3	0.1	0.6	–	–
Bigeye tuna deoMb solution*		13.5	0.2	104.7	−6.0	98.9	−0.5	99.4	1.1	–	–
Bigeye tuna oxyMb solution*		5.1	7.1	9.4	78.2	94.7	−0.6	−0.1	100.7	–	–
Bigeye tuna lot 1	0 °C for 1 day	10.2 ± 1.7	23.3 ± 1.4	9.0 ± 0.1	64.7 ± 1.5	97.0 ± 0.1	22.1 ± 1.9	11.5 ± 0.2	66.8 ± 2.0	0.089 ± 0.001	0.068 ± 0.003
	0 °C for 2 days	11.2 ± 1.1	24.4 ± 1.3	8.3 ± 0.4	64.2 ± 1.6	96.9 ± 0.1	23.5 ± 1.6	10.5 ± 0.6	66.0 ± 2.3	0.074 ± 0.003	0.060 ± 0.002
	0 °C for 4 days	11.9 ± 1.0	24.3 ± 0.8	7.4 ± 0.5	65.2 ± 1.2	96.9 ± 0.1	23.0 ± 1.0	9.4 ± 0.8	67.6 ± 1.8	0.079 ± 0.003	0.065 ± 0.003
	0 °C for 7 days	18.7 ± 1.0	30.7 ± 1.2	7.8 ± 0.2	58.7 ± 1.3	97.2 ± 0.1	30.5 ± 1.4	10.1 ± 0.4	59.4 ± 1.7	0.085 ± 0.001	0.069 ± 0.001
Bigeye tuna lot 2	0 °C for 1 day	11.2 ± 0.1	24.6 ± 0.1	8.8 ± 0.1	63.6 ± 0.1	97.0 ± 0.0	23.9 ± 0.1	11.2 ± 0.2	64.9 ± 0.3	0.090 ± 0.007	0.067 ± 0.004
	0 °C for 2 days	15.9 ± 0.6	28.9 ± 0.6	8.4 ± 0.2	59.9 ± 0.7	97.2 ± 0.0	28.8 ± 0.8	10.9 ± 0.3	60.4 ± 1.1	0.077 ± 0.001	0.061 ± 0.001
	0 °C for 4 days	14.7 ± 0.5	27.2 ± 0.6	7.5 ± 0.5	62.3 ± 1.0	97.0 ± 0.1	26.7 ± 0.9	9.9 ± 1.0	63.4 ± 1.7	0.082 ± 0.005	0.067 ± 0.003
	0 °C for 7 days	17.1 ± 0.3	29.1 ± 0.7	6.6 ± 0.5	61.4 ± 1.2	97.1 ± 0.1	28.1 ± 1.1	8.1 ± 0.9	63.8 ± 2.0	0.085 ± 0.003	0.071 ± 0.000
Bigeye tuna lot 3	5 °C for 1 day	31.1 ± 0.7	42.6 ± 0.8	6.3 ± 0.5	49.2 ± 1.2	98.1 ± 0.1	42.4 ± 1.0	8.7 ± 0.7	48.8 ± 1.6	0.093 ± 0.004	0.081 ± 0.002
	5 °C for 2 days	40.4 ± 0.3	51.1 ± 0.2	3.1 ± 0.4	44.2 ± 0.5	98.4 ± 0.0	50.6 ± 0.3	5.1 ± 0.4	44.3 ± 0.7	0.083 ± 0.002	0.080 ± 0.003
Bigeye tuna lot 4	Fresh	−5.9 ± 0.8	6.5 ± 0.8	5.6 ± 0.3	84.0 ± 1.1	96.1 ± 0.1	−2.3 ± 1.1	4.0 ± 0.6	98.3 ± 1.7	0.162 ± 0.004	0.178 ± 0.006
	5 °C for 1 day	−1.8 ± 0.3	10.5 ± 0.4	5.4 ± 0.3	80.5 ± 0.6	96.4 ± 0.1	2.8 ± 0.5	4.3 ± 0.5	92.9 ± 1.0	0.154 ± 0.003	0.165 ± 0.005
	5 °C for 4 days	10.1 ± 0.3	22.8 ± 0.4	4.6 ± 0.1	69.7 ± 0.5	97.1 ± 0.1	18.4 ± 0.5	4.4 ± 0.3	77.2 ± 0.8	0.169 ± 0.001	0.178 ± 0.001
	5 °C for 5 days	14.5 ± 0.1	25.4 ± 0.2	3.4 ± 0.1	68.2 ± 0.2	97.0 ± 0.0	21.3 ± 0.2	3.1 ± 0.1	75.6 ± 0.3	0.161 ± 0.001	0.176 ± 0.000
	5°Cfor 10 days	36.1 ± 0.5	41.7 ± 0.6	5.2 ± 0.5	50.7 ± 1.0	97.6 ± 0.1	41.4 ± 0.7	6.5 ± 0.7	52.1 ± 1.4	0.160 ± 0.006	0.166 ± 0.008
Bigeye tuna lot 5	Fresh	8.3 ± 0.5	20.9 ± 0.5	5.9 ± 0.3	70.2 ± 0.8	97.0 ± 0.1	16.7 ± 0.8	6.1 ± 0.5	77.2 ± 1.2	0.139 ± 0.002	0.129 ± 0.002
	5 °C for 1 day	14.3 ± 0.3	26.4 ± 0.4	4.7 ± 0.2	66.0 ± 0.5	97.2 ± 0.0	23.2 ± 0.6	5.0 ± 0.3	71.8 ± 0.8	0.144 ± 0.001	0.139 ± 0.003
	5 °C for 3 days	26.1 ± 0.3	37.6 ± 0.3	3.2 ± 0.3	56.8 ± 0.6	97.6 ± 0.1	36.0 ± 0.4	4.0 ± 0.5	60.0 ± 0.9	0.133 ± 0.002	0.130 ± 0.006
	5 °C for 4 days	28.5 ± 0.7	39.9 ± 0.7	2.9 ± 0.4	55.0 ± 1.1	97.7 ± 0.1	38.4 ± 0.9	3.8 ± 0.6	57.8 ± 1.5	0.134 ± 0.004	0.130 ± 0.003
	5 °C for 5 days	37.3 ± 0.0	47.9 ± 0.1	0.7 ± 0.1	49.5 ± 0.1	98.0 ± 0.0	46.7 ± 0.0	1.8 ± 0.1	51.6 ± 0.1	0.129 ± 0.000	0.131 ± 0.000
	5 °C for 7 days	69.5 ± 0.5	70.6 ± 0.6	6.7 ± 0.3	21.8 ± 0.4	99.0 ± 0.0	71.9 ± 0.5	10.6 ± 0.3	17.4 ± 0.4	0.148 ± 0.003	0.121 ± 0.003
Bluefin tuna lot 1	Fresh	7.8 ± 0.3	18.6 ± 0.3	11.9 ± 0.1	66.5 ± 0.3	96.9 ± 0.0	13.6 ± 0.6	11.2 ± 0.2	75.1 ± 0.6	0.261 ± 0.002	0.282 ± 0.003
	5 °C for 1 day	10.7 ± 0.4	21.2 ± 0.4	10.5 ± 0.3	65.3 ± 0.5	97.0 ± 0.1	16.7 ± 0.5	9.9 ± 0.4	73.5 ± 0.8	0.250 ± 0.004	0.272 ± 0.003
	5 °C for 3 days	24.9 ± 0.2	35.5 ± 0.2	6.8 ± 0.2	55.2 ± 0.3	97.6 ± 0.0	33.4 ± 0.2	6.7 ± 0.2	59.9 ± 0.4	0.255 ± 0.013	0.278 ± 0.012
	5 °C for 4 days	30.3 ± 0.1	41.3 ± 0.2	6.4 ± 0.7	50.2 ± 0.8	97.9 ± 0.1	40.0 ± 0.6	7.3 ± 1.3	52.6 ± 2.0	0.258 ± 0.007	0.269 ± 0.003
	5 °C for 5 days	43.2 ± 0.6	53.3 ± 0.5	4.7 ± 0.2	40.4 ± 0.3	98.4 ± 0.0	52.4 ± 0.5	5.6 ± 0.2	42.0 ± 0.3	0.238 ± 0.000	0.252 ± 0.002
	5 °C for 7 days	71.3 ± 0.3	79.5 ± 0.2	5.0 ± 0.1	15.1 ± 0.3	99.5 ± 0.0	78.4 ± 0.2	7.3 ± 0.2	14.3 ± 0.3	0.271 ± 0.004	0.264 ± 0.005

* Results for the solutions of bigeye tuna Mb were calculated from the data of Matsuura and Hashimoto (1955), which are summarised in Table 3.

3. Results and discussion

3.1. Estimation of the %metMb in aqueous tuna meat extracts using equations derived from the absorption spectra of horse Mb

Equations that have been widely employed in determining the relative concentrations of Mb derivatives in aqueous meat extracts are summarised in Table 1. All equations were basically derived from the absorption spectra of horse Mb using the method of Krzywicki (1979, 1982). In addition, the absorbance at 525 nm, i.e., the isobestic point for all three Mb derivatives, has been selected to determine total Mb concentration in all previously reported equations. In Eqs. (1)–(3), the isobestic point at 572 nm, where the extinction coefficients of horse oxyMb and deoMb are equivalent, has been selected to determine the relative concentrations of Mb derivatives. Absorbance at 700 and 730 nm have been selected for a correction for turbidity (Goldbloom & Brown, 1966), which is difficult to remove from meat extracts. On the other hand, Eq. (4) was derived on the basis of the arbitrary selection of the reference wavelengths (Krzywicki, 1982). Although Eq. (4) have been employed in many studies (Faustman & Phillips, 2001; Badr, 2007), Tang et al. (2004) have recently reported that the set of equations (4) have generated negative values for some Mb redox forms and the percentages of total Mb have deviated from 100%. As a result, Tang et al. (2004) developed a new set of equations with the selection of wavelengths representing absorption maximum for each of the three derivatives, as shown in Eq. (5).

Since Eqs. (1) and (5) have been widely employed to estimate the %metMb for various kinds of fish samples (Benjakul & Bauer, 2001; Chen, 2003; Chaijan et al., 2005, 2006, 2007; Viriyarattanasak et al., 2008), both equations were selected to estimate and compare %metMb in aqueous tuna extracts in this study. Additionally, a comparative analysis of aqueous beef extracts was also performed. The calculated results with Eqs. (1) and (5) for tuna samples are shown in Table 2. In the same aqueous tuna extract, %metMb calculated with equation (1) was considerably lower than that calculated with Eq. (5) for all tuna samples, regardless of tuna type and preservation conditions. The plot between the %metMb calculated with Eqs. (1) and (5) is shown in Fig. 2A. An obvious relationship was seen between %metMb calculated using Eq. (1) and that using Eq. (5) deviated from unity. Moreover, the results from the calculation with Eq. (5) showed that the sum of the percentages of the three Mb derivatives (%total Mb) ranged from 96.1% to 99.5% (Table 2), and these %total Mb increased with a corresponding increase in %metMb (Fig. 2B). In comparison, the results for beef samples show that the %metMb calculated with Eq. (1) was almost equivalent to that calculated with Eq. (5) (Fig. 2A). In addition, the %total Mb was approximately 100% for all beef samples (Fig. 2B). These results suggest that both Eqs. (1) and (5) would be appropriate for determining the %metMb in aqueous beef extracts with similar precision and accuracy. On the other hand, differences in the evaluating equations resulted in different estimations of %metMb even in the same aqueous tuna sample extracts. This may provide different interpretations of the obtained results and also in evaluations of tuna meat quality.

Other than in the aqueous meat extracts, equations (1) and (5) were also employed to estimate the %metMb and %total Mb in pure solutions of bigeye tuna Mb for each of the derivatives. The millimolar extinction coefficients at the wavelengths, shown in Eqs. (1) and (5), for the three Mb derivatives in bigeye tuna were taken from Fig. 1B and summarised in Table 3. In comparison, the millimolar extinction coefficients, which were actually used in the establishment of Eqs. (1) and (5), for three Mb derivatives of horse Mb are also summarised in Table 3. These extinction coefficients for both horse and bigeye tuna Mb were calculated from the application of Lambert–Beer Law (Bowen 1949; Matsuura & Hashimoto, 1955; Tang et al., 2004). However, the extinction coefficients of horse Mb were calculated on the basis of Mb concentration, while those for tuna Mb were calculated based upon equivalent iron. From Table 3, it is obvious that the extinction coefficients of metMb and deoMb for tuna were close to those for horse. However, the extinction coefficients of oxyMb in tuna were quite different from those in horse, particularly the extinction coefficients at 572 and 582 nm (Table 3). The data in Table 3 was employed to calculate the %metMb and %total Mb in pure solutions of bigeye tuna Mb for each derivative and the calculated results are shown in Table 2. The results show that for pure oxyMb and metMb solutions in bigeye tuna, the %metMb estimated by equation (1) were lower than those estimated by Eq. (5). This result is in accordance with the result of tuna extracts, and thus would explain why the %metMb calculated by equation (1) was significantly lower than those calculated by Eq. (5) for tuna extracts (Fig. 2A, Table 2). On the other hand, the %total Mb for metMb and deoMb solutions of bigeye tuna was approximately 100%, while the %total Mb for oxyMb solution was 94.7%. This result is also in accordance

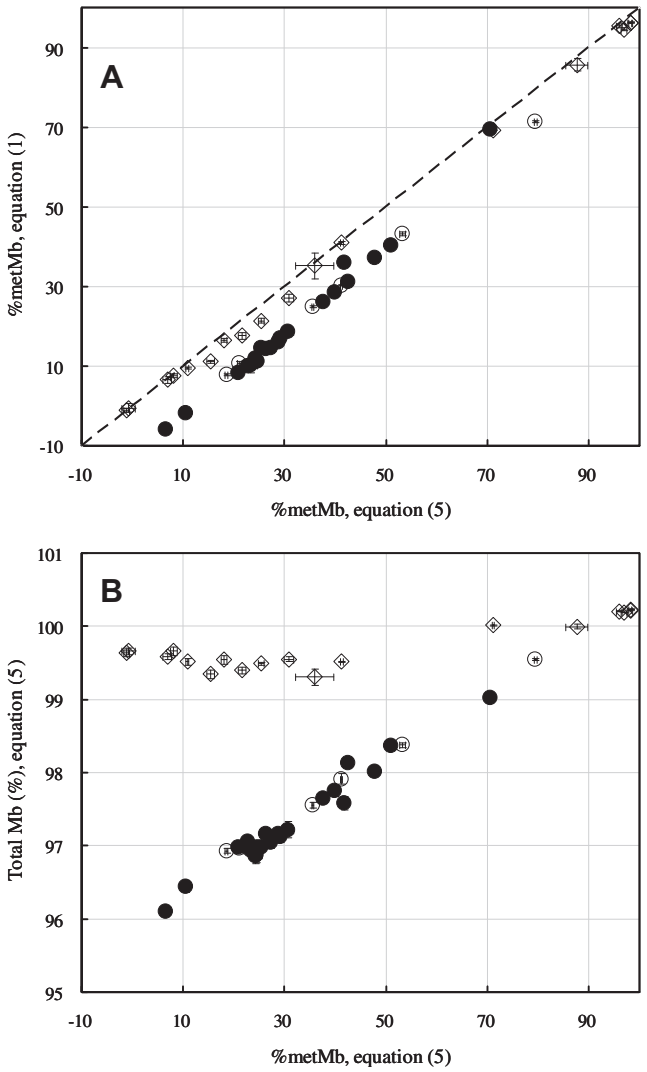

Fig. 2. Comparison of the %metMb estimated by Eqs. (1) and (5) (A), and the %metMb and %totalMb estimated by Eq. (5) (B). Closed and open circles represent data for bigeye and bluefin tuna samples, respectively. Open diamonds represent data for beef samples. The dashed line in (A) indicates the expected linear relation when the slope is 1.

with the results in tuna extracts, and thus would explain why the %total Mb increased with a corresponding increase in %metMb for tuna extracts (Fig. 2B). The agreement in the results for the pure solution of tuna Mb and aqueous tuna extracts demonstrates that the difference in the absorption spectra for horse and tuna Mb is sensitive for determining the relative concentrations of Mb derivatives in tuna samples using equations derived from horse Mb.

Although the data is not shown here, we found that Eq. (4) gave negative %metMb values for fresh fish meat samples and the %total Mb ranged from 72.2% to 91.6%. These results are in agreement with Tang et al. (2004), who also reported negative values of some Mb derivatives and that the %total Mb deviated from 100% for pure equine Mb solution when calculated using Eq. (4). Therefore, this strongly suggests that Eq. (4) may not be appropriate for estimating the relative concentrations of Mb derivatives in aqueous tuna extracts.

From the overall results, it is suggested that it may not be appropriate to determine the relative concentrations of Mb derivatives in tuna samples using the previously reported equations derived from horse Mb. Therefore, a new set of equations, which were derived from absorption spectra of bigeye tuna Mb (the data of Matsuura & Hashimoto, 1955), was developed.

3.2. Establishment of equations

A new set of equations was established according to the method of Krzywicki (1982). Since meat extract typically contains three Mb derivatives, i.e., deoMb, oxyMb, and metMb, the following set of three equations must be solved in calculating the Mb derivatives:

$$A_{\lambda 1} = C_{\text{deoMb}} E_{\lambda 1}^{\text{mM,deoMb}} + C_{\text{oxyMb}} E_{\lambda 1}^{\text{mM,oxyMb}} + C_{\text{metMb}} E_{\lambda 1}^{\text{mM,metMb}} \quad (1)$$

$$A_{\lambda 2} = C_{\text{deoMb}} E_{\lambda 2}^{\text{mM,deoMb}} + C_{\text{oxyMb}} E_{\lambda 2}^{\text{mM,oxyMb}} + C_{\text{metMb}} E_{\lambda 2}^{\text{mM,metMb}} \quad (2)$$

$$A_{\lambda 3} = C_{\text{deoMb}} E_{\lambda 3}^{\text{mM,deoMb}} + C_{\text{oxyMb}} E_{\lambda 3}^{\text{mM,oxyMb}} + C_{\text{metMb}} E_{\lambda 3}^{\text{mM,metMb}} \quad (3)$$

where A is an absorbance at a constant wavelength (λ); C is the millimolar concentration of each Mb derivative and E_{mM} is the millimolar extinction coefficient at the specific wavelength for each Mb derivative. To establish accurate equations, the wavelengths were selected from the absorption maximum for each of the three Mb derivatives (Benesch, Benesch, & Yung, 1973; Tang et al., 2004). Therefore, $\lambda 1$, $\lambda 2$, and $\lambda 3$ were 500, 540, and 555 nm, respectively, according to the absorption spectra of bigeye tuna Mb in Fig. 1B. The wavelengths of 500, 540, and 555 nm were the wavelengths of maximal absorption for metMb, oxyMb, and deoxMb, respectively. The millimolar extinction coefficients at these chosen wavelengths, which are shown in Table 4, were incorporated into Eqs. (6)–(8), and the solutions were presented as follows:

$$C_{\text{metMb}} = -0.010A_{555} - 0.057A_{540} + 0.150A_{500}$$

$$C_{\text{deoMb}} = 0.173A_{555} - 0.109A_{540} - 0.003A_{500}$$

$$C_{\text{oxyMb}} = -0.116A_{555} + 0.165A_{540} - 0.052A_{500}$$

$$C_{\text{total}} = C_{\text{oxyMb}} + C_{\text{deoMb}} + C_{\text{metMb}}$$

The absorption spectra of tuna Mb did not show the isobestic point, where the extinction coefficients of the three Mb derivatives are equivalent (Matsuura & Hashimoto, 1955). Therefore, the calculation of the relative concentrations of Mb derivatives was based on the sum of individually calculated Mb derivative concentrations (C_{total}).

Table 3
Millimolar extinction coefficients at the wavelengths selected to establish Eqs. (1) and (5) for the three Mb derivatives of bigeye tuna ((a) Matsuura & Hashimoto, 1955] and horse [(b) Bowen, 1949; (c) Tang et al., 2004).

λ (nm)	deoMb		oxyMb		metMb	
	Bigeye tuna (a)	Horse	Bigeye tuna (a)	Horse	Bigeye tuna (a)	Horse
582	7.10	8.64 (c)	8.90	14.37 (c)	2.80	3.29 (c)
572	9.70	10.60 (b)	12.50	10.60 (b)	2.82	3.00 (b)
557	12.20	12.30 (c)	9.22	9.47 (c)	3.25	4.05 (c)
525	7.70	7.60 (b)	9.30	7.60 (b)	6.75	7.60 (b)
503	4.90	5.05 (c)	6.10	5.73 (c)	8.80	9.84 (c)

(a) Data of Matsuura and Hashimoto (1955) were calculated on the basis of equivalent iron content.
(b) Data of Bowen (1949) were calculated on the basis of Mb concentration.
(c) Data of Tang et al. (2004) were calculated on the basis of Mb concentration.

Table 4
Millimolar extinction coefficients ($\varepsilon_{\lambda}^{*}$, /mM/cm) for myoglobin according to Matsuura and Hashimoto (1955).

Myoglobin form	Wavelength (nm)		
	500	540	555
metMb	8.90	5.30	3.50
oxyMb	6.13	14.60	9.30
deoMb	4.63	10.13	12.24

* Data of Matsuura and Hashimoto (1955) were calculated on the basis of equivalent iron content.

3.3. Comparison of the results obtained by the cyanmetmyoglobin method and by calculation from the proposed equations

The new set of proposed equations was employed to estimate the relative concentrations of the three Mb derivatives and total Mb concentration in aqueous tuna extracts, as shown in Table 2. To validate the proposed equations, total Mb concentration in these tuna extracts was also determined by the cyanmetMb method, and the obtained results were compared with those estimated by the proposed equations (Table 2). The results show a good linear relationship between the total Mb concentrations determined by the cyanmetMb method and those estimated by our proposed equations ($R^2 = 0.984$) (Fig. 3). Therefore, this suggests that the new set of proposed equations can be applied to the determination

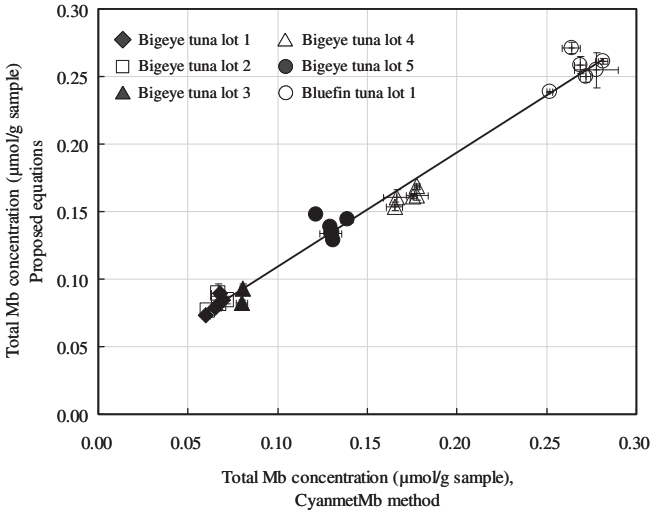

Fig. 3. Comparison of results obtained by the cyanmetMb method and by the calculation from the proposed equations for tuna meat extracts.

of the relative concentrations of Mb derivatives and also the total Mb concentration in aqueous tuna extracts. Since there are some differences in the absorption maxima and minima depending on the kind of fish sample (Amano & Tsuyuki, 1975; Matsuura & Hashimoto, 1955; Yamaguchi, Takeda, Ogawa, & Hashimoto, 1979), the application of the developed equations to determine the relative concentrations of Mb derivatives for other kinds of fish samples is now under evaluation.

4. Conclusion

The present study reports that differences in the previously reported equations, used for estimating the %metMb in aqueous meat extracts, results in differences in the estimated values for tuna samples. In contrast, differences in the results estimated by these different equations were negligible for beef samples. Since the previously reported equations have been derived from the absorption spectra of horse Mb, it is suggested that the difference in the absorption spectra of horse and tuna Mb might be responsible for the discrepancy in the results of tuna samples. Therefore, in the present study a new set of equations was established from the absorption spectra of bigeye tuna Mb, reported by Matsuura and Hashimoto (1955). The total Mb concentrations estimated by our proposed equations were in good agreement with the results obtained by the conventional cyanmetMb method ($R^2 = 0.984$). Therefore, a new set of equations was proposed to determine the relative concentrations of Mb derivatives and total Mb concentration in aqueous extracts of tuna meat samples.

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