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pH-Induced Shift in Hemoglobin Spectra: A Spectrophotometeric Comparison of Atlantic Cod (*Gadus morhua*) and Mammalian Hemoglobin

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ABSTRACT: Due to a pH-sensitive effect in many fish hemoglobins (Hb), analytical errors may occur when mammalian Hb is used as a standard in quantitative spectrophotometric multicomponent analysis of fish blood. The aim of this work was to examine differences in the optical spectra of mammalian (human) and fish (farmed Atlantic cod) Hb subjected to pH 7.4 and 6.5. The absorption spectra of the common derivatives, deoxy- (HHb), oxy- (OHb), carboxy- (COHb), and methemoglobin (metHb), were determined in the spectral range of 450−700 nm. The metHb spectra of fish differed considerably from the corresponding human Hb spectra, whereas only minor differences in OHb, HHb, and COHb were found. Cod Hb was significantly ($P < 0.05$) influenced by a drop in pH compared to mammalian Hb. This resulted in deoxygenation of the Hb and increased autoxidation. For human Hb, a pH-independent isosbestic point in the spectra of OHb, HHb, and metHb at 523 nm was found. This isosbestic point was not found in the absorption spectra of cod Hb. In conclusion, spectra of cod metHb and human metHb behave differently. This must thus be taken into account in spectrophotometric multicomponent analysis. Ideally, Hb in muscle or blood should be determined by comparison to a standard made from the same species.

KEYWORDS: Blood, hemoglobin, fish, optical absorbance spectra, spectrophotometry, autoxidation

INTRODUCTION

The natural color of blood is largely due to the concentration and chemical state of heme iron and can dictate the color of fish muscle, causing the product to be downgraded or rejected.[1−3] Poor bleeding has been registered as blood spots or blood effusions in the fillets, and labor-intensive procedures such as candling and manual inspection have traditionally been used to detect blood residuals in fish.[3−6] It is important to note that both candling and manual inspection involve subjective evaluation and have a high level of inaccuracy.[3,7] For a more accurate measurement of blood content in meat and fish, chemical estimation of hemoglobin (Hb) has been applied,[3] but chemical methods are also labor-intensive. They involve toxic chemicals and are destructive to the product. Therefore, these methods may be considered impractical and not useful for industrial online production.[8]

The focus on instrumental detection based on optical properties of the muscle for rapid determination of fish quality is increasing.[1,7−12] The evolution in computer technology and multivariate computer software during the past decades has made a powerful analytical tool out of spectrophotometric multicomponent analysis. This is because only small differences in the absorbance spectra of individual compounds are sufficient for quantitative analysis. Advantages of using spectrophotometric multicomponent analysis include the ability to measure components in materials with little or no sample preparation; that is, it is nondestructive and rapid and may thus be applied industrially.[13−17]

To predict the chemical composition of food, using spectrophotometric multicomponent analysis, the reference method for calibration is decisive, and numerous calibrations have been developed for this task.[16] In spectrophotometry of Hb derivatives, it has been assumed that the absorptivities determined for human blood also would be valid for blood of other mammals, due to insignificant variations in the optical Hb spectra among mammalian species.[18] However, to avoid contamination with human pathogens, bovine blood is used as a substitute for human blood in the production of reference materials and quality assessment in multicomponent analysis of Hb. The most common Hb derivatives in blood, oxy- (OHb), carboxy- (COHb), deoxy- (HHb), and methemoglobin (metHb), can be identified at wavelengths from 476 to 651 nm by visible spectroscopy. However, temperature and pH can influence the optical spectra.[18−20]

It is known that, among vertebrates, fish display the most extensive presence of multiple Hb components, which show considerable differences in amino acid sequence and functional properties. This is probably due to the enormous plasticity in the selection of environments and the capacity of some species to cope with different conditions of temperature, pressure, salinity, pH, and oxygen availability.[21−25] It is also known that in some fish species, part of the Hb in the blood is extremely pH sensitive, and this is commonly known as the Root effect. The Root effect decreases the oxygen-carrying capacity rapidly when the pH is reduced, and even at a high partial pressure of oxygen, parts of the Hb remain deoxygenated (HHb).[23−25] The Root effect does not occur in mammalian Hb. However, mammalian Hb and fish Hb have another effect in common, known as the Bohr effect, which generally alters the oxygen-binding properties of hemoglobins in

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response to changes in both partial pressure of oxygen and pH.[22,23]

In fish, muscle pH rapidly decreased from pH 7.5 to 6.5 within hours after death, reaching ultimate muscle pH after 24 h.[26,27] Anaerobic muscle metabolism is the primary cause of reduced pH. However, the metabolism of red and white blood cells can also contribute to carbon dioxide and lactate production in blood, resulting in the rapid reduction of blood pH.[28] Hence, both the rapid reduction of blood pH and the Root effect of Hb in fish can result in pH-induced analytical errors. This is particularly relevant when mammalian Hb is used as a standard for quantitative spectrophotometric multicomponent analysis of fish Hb. Especially, pH-induced changes in the visible range (450−700 nm) are crucial, because this is the range that contains most information regarding the presence of Hb.[8] Even if these differences are small, they can be of importance because of the sensibility of a spectrophotometric multicomponent analysis,[29,30] which in turn may result in a significant under- or overestimation of Hb content.

The aim of this study was to investigate pH-induced changes in the optical absorbance spectra of fish and mammalian OHb, COHb, HHb, and metHb in the visible range (450−700 nm) and evaluate the analytical significance of the Root effect.

MATERIALS AND METHODS

Fish. Maintenance fed (Amber, Skretting Ltd., Norway) farmed Atlantic cod (*Gadus morhua*) was acquired from the Tromsø Aquaculture Research Station (Kårvika, Tromsø) in March 2009. The juveniles (50 g) were supplied by LofiTorsk AS (Steine, Lofoten, Norway) to the aquaculture station in November 2007. The fish ($n = 5$) used was grown to commercial size (3616 ± 461 g, 65 ± 3.2 cm) with a mean condition factor (CF) of 1.3. The CF was calculated according to the formula CF = [total body weight (g)/fork length (cm)^3] × 100.

The fish was rapidly and carefully netted from the cage and immediately killed with a blow to the head. Full loss of consciousness or death was assumed, as the fish showed no behavioral response after the blow. Within 5 min after killing, muscle pH was determined using a WTW pH-meter (pH 330; Wissenschaftlich-Technische Werkstatten GmbH, Weilheim, Germany), equipped with a Hamilton double-pore glass electrode (Hamilton Bonaduz AG, Bonaduz, Switzerland). The electrode was inserted into the loin between the dorsal fin and the lateral line. Muscle pH was also determined after 24 h and 7 days post-mortem.

Fish and Mammalian Blood Collection. The fish blood was collected ($n = 5$) immediately after killing. Blood was drawn from the caudal vein using a 10 mL syringe with a 20 G/0.9 mm needle. Sodium heparin (100 international units (IU) mL^{-1} of blood) was added to prevent coagulation.

For practical reasons human blood was chosen as the mammalian Hb source. Venous blood was drawn from the forearm of presumably nonsmoking healthy adult donors ($n = 3$). About 10 mL of blood per individual (fish and mammalian) was collected. The sampling of human blood was performed in compliance with the appropriate laws and institutional guidelines for the University of Tromsø, Norway. A statement of informed consent was also obtained from each donor.

Experimental Design. Fish and human blood samples were processed into clear hemolysate and further divided into two phosphate-buffered Hb solutions with pH 6.5 and 7.4. The phosphate-buffered Hb solutions were then stored at 2−4 °C before chemical quantification, tonometry, and spectrophotometric measurements of Hb were carried out.

Preparation of a Clear Hemoglobin Solution. By modifying an earlier described method of preparing mammalian Hb,[30] a clear solution of Hb was prepared. All heparinized blood samples were stored on ice for 2−3 h before separation of erythrocytes by centrifugation (900*g*, 30 min). The plasma was removed, and the erythrocytes were washed three times with ice-cold saline (9 g L^{-1} NaCl) solution. A stock solution of Hb was prepared by resuspending the packed erythrocytes in saline. The total Hb concentration was kept between 100 and 150 mg mL^{-1} (2.5 mL of packed erythrocytes and 4.0 mL of saline solution). Hemolysis was performed in saline solution containing 2% Triton X-100, in a ratio of 1:4 (stock solution and saline). To obtain a clear hemolysate, the hemolyzed stock solution was centrifuged for 20 min at 8000*g* at 4 °C and filtered through a Whatman no. 1 filter paper.

Phosphate-Buffered Hemoglobin Solution. Two 0.01 M phosphate-buffered saline (PBS) solutions (pH 7.4 and 6.5) were prepared from disodium hydrogen phosphate monohydrate ($Na_2HPO_4 \cdot H_2O$) and sodium dihydrogen phosphate heptahydrate ($NaH_2PO_4 \cdot 7H_2O$). Hydrochloric acid (HCl) and NaOH were added to adjust the pH, and the osmolality of the solutions was adjusted to 330 mOsmol by adding NaCl. The Hb solutions were diluted with PBS into two 50 mL centrifuge tubes (pH 7.4 and 6.5) to a Hb concentration of 0.05 mmol L^{-1}. This ensured that the Hb absorption spectra were below an optical density (OD) of 2.0. The diluted Hb solutions were then stored in the refrigerator at 2.0−4.0 °C for 60 min before tonometry.

Quantification of Hb. A modified Hornsey method[31] for heme pigment determination was used to quantify the Hb content in the phosphate-buffered saline (PBS) Hb solutions. A volume of 0.5 mL of PBS Hb solution was transferred into a 50 mL centrifuge tube and diluted with 4.0 mL of cold PBS. Acetone (20 mL) and 0.5 mL of 37% HCl were added to the centrifuge tube, and the mixture was vortexted for 15 s and then stored in a refrigerator at 4 °C. After 60 min, the mixture was filtrated through a Whatman no. 1 filter paper. The filtrate was centrifuged at 10000*g* for 15 min, and spectrophotometric measurements were performed at 640 nm. The amount of heme pigment was calculated using a standard curve made from bovine Hb (Sigma-Aldrich). The amount of Hb in the sample was calculated by multiplying the concentration of Hb (g L^{-1}) in the sample to the sample volume processed (0.5 mL).

Tonometry of Fish and Mammalian Hemoglobin Solution. Prior to tonometry, 5 mL of the diluted Hb solutions was drawn into antifoam-treated 6 mL plastic syringes (catalog no. T310; RNA Medical, Devens, MA). The Hb derivatives OHb, COHb, and HHb were prepared in a three-channel bubble-type syringe tonometer (Equilibrator, catalog no. T1004S; RNA Medical).

To prevent denaturation of the Hb, the plastic syringes were kept in ice (0−1 °C) during tonometry. Gas was delivered to the samples through a humidifying buffer solution (R1210, LOT 710016; RNA Medical). All Hb solutions were treated with gaseous mixtures for 40 min before spectrophotometric measurements.

The OHb was prepared with a gaseous mixture containing 95% oxygen (O_2) and 5% carbon dioxide (CO_2). The COHb was prepared with a gaseous mixture containing 95% nitrogen (N_2) and 5% carbon monoxide (CO). The HHb was prepared with a gaseous mixture containing 95% nitrogen (N_2) and 5% carbon dioxide (CO_2). After tonometry, 1−2 mg of sodium dithionite ($Na_2S_2O_4$) was added to the erythrolysate to avoid reoxygenation of HHb when exposed to air. The metHb was prepared by adding 1−2 mg of solid potassium ferricyanide ($K_3Fe(CN)_6$) into the diluted Hb solution. Furthermore, the solution was mixed for 30 s before storage in the refrigerator at 2−4 °C for 10 min to convert all of the Hb derivatives to metHb.

Spectrophotometric Measurements. Prior to spectrophotometric measurements, all Hb solutions were filtered through a 0.45 μm standard 25 mm Acrodisc syringe filter (Pall Life Sciences, Port Washington, NY) into a 3.5 mL standard quartz cuvette with a 10 mm optical path length. The absorbance scan was performed in the 450−700 nm wavelength range at room temperature using a PC-controlled

Table 1. Estimated Percentage of Oxyhemoglobin (OHb), Deoxyhemoglobin (HHb), Carboxyhemoglobin (COHb), and Methemoglobin (metHb)[a]

	(A) Atlantic Cod (n = 5) Hemolysate					
tonometry cod Hb	95% O_2/5% CO_2		95% N_2/5% CO		95% N_2/5% CO_2 (sodium dithionite)	
pH	7.4	6.5	7.4	6.5	7.4	6.5
OHb %	86.4 ± 1.8	56.6 ± 2.8	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
COHb %	0.0 ± 0.0	0.0 ± 0.0	98.1 ± 0.6	94.6 ± 0.9	0.0 ± 0.0	0.0 ± 0.0
HHb %	9.4 ± 1.3	29.5 ± 1.9	0.0 ± 0.0	0.0 ± 0.0	98.3 ± 0.4	96.8 ± 1.1
metHb %	4.2 ± 2.2	13.9 ± 2.2	1.9 ± 1.4	5.4 ± 1.2	1.7 ± 1.6	3.2 ± 2.0
R^2	0.996	0.994	0.999	0.999	0.999	0.999
RMSEP	0.316	0.149	7.57×10^{-2}	7.42×10^{-2}	5.70×10^{-7}	4.73×10^{-2}
	(B) Human (n = 3) Hemolysate					
tonometry human Hb	95% O_2/5% CO_2		95% N_2/5% CO		95% N_2/5% CO_2 (sodium dithionite)	
pH	7.4	6.5	7.4	6.5	7.4	6.5
OHb %	93.8 ± 0.5	91.3 ± 0.6	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
COHb %	0.0 ± 0.0	0.0 ± 0.0	97.4 ± 0.8	95.6 ± 0.5	0.0 ± 0.0	0.0 ± 0.0
HHb %	3.8 ± 0.8	5.5 ± 0.8	0.0 ± 0.0	0.0 ± 0.0	98.2 ± 0.4	97.5 ± 0.9
metHb %	2.4 ± 1.1	3.2 ± 1.3	2.6 ± 1.3	4.4 ± 1.2	1.8 ± 0.8	2.5 ± 1.1
R^2	0.999	0.999	0.999	0.999	0.999	0.999
RMSEP	3.56×10^{-6}	7.28×10^{-2}	9.53×10^{-2}	9.12×10^{-2}	3.93×10^{-2}	6.19×10^{-2}

[a] The three different hemolysates were prepared with gaseous mixtures containing 95% oxygen (O_2) and 5% carbon dioxide (CO_2), 95% nitrogen (N_2) and 5% carbon monoxide (CO), and 95% nitrogen (N_2) and 5% carbon dioxide (CO_2). The percentage (mean ± SEM) of the hemoglobin derivatives were determined by spectral deconvolution and multiple linear least-squared curve fitting procedures. The fitting of the spectral data is given by the coefficient of determination (R^2) between measured Hb spectra and predicted Hb spectra. The prediction error is given by the RMSEP.

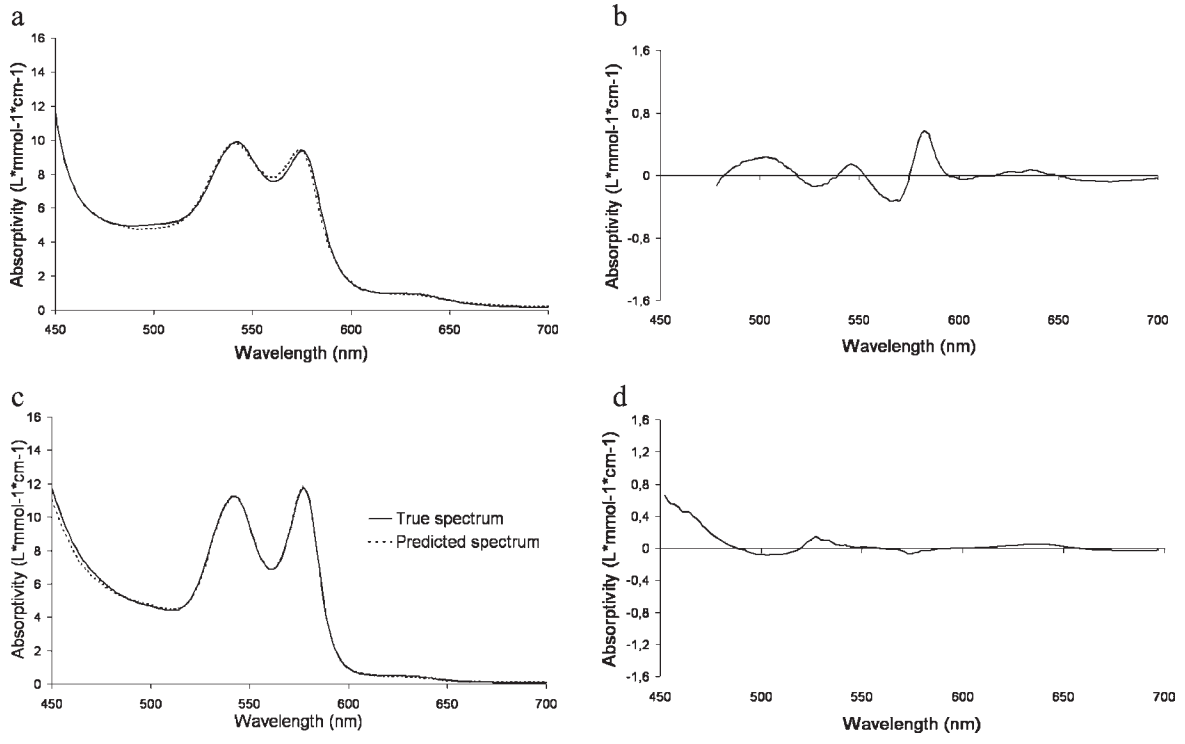

Figure 1. Representative results from the multiple linear last-square curve fitting procedure in the spectral region of 450−700 nm: (a) experimental (true) spectrum of the oxygenated Atlantic cod hemolysate at pH 6.5; (b) differences between true spectrum and fitted curve of the oxygenated Atlantic cod hemolysate; (c) experimental (true) spectrum of the oxygenated human hemolysate at pH 6.5 (the dotted line in all panels gives the fitted curve); (d) differences between the true spectrum and the fitted curve of the oxygenated human hemolysate. Both panels b and d are enlarged 10 times.

dual-beam spectrophotometer (SPEKOL 2000, Analytik Jena AG, Jena, Germany), with bandwidth set to 1.0 nm.

Statistics and Calculations. Results are presented as mean ± standard error of the mean (SEM). The statistical significance between

Table 2. Estimated Millimolar Absorptivities of 100% Atlantic Cod (n = 5) and Human (n = 3) Oxyhemoglobin (OHb), Deoxyhemoglobin (HHb), and Carboxyhemoglobin (COHb), Determined by a Spectral Deconvolution and Multiple Linear Least-Square Curve Fitting Procedure[a]

λ (nm)	OHb	cod	human
508	min	4.84 ± 0.05	
510	min		4.76 ± 0.07*
540	max	13.44 ± 0.03	
542	max		13.27 ± 0.04*
560	min	8.08 ± 0.03	
561	min		8.19 ± 0.03*
575	max	14.10 ± 0.04	
577	max		14.12 ± 0.04*
λ (nm)	COHb	cod	human
493	min	5.18 ± 0.05	5.34 ± 0.06*
539	max	13.07 ± 0.03	12.82 ± 0.03
555	min	10.36 ± 0.03	10.43 ± 0.03
569	max	12.69 ± 0.04	12.85 ± 0.04
λ (nm)	HHb		
477	min	3.11 ± 0.02	3.11 ± 0.04
556	max	12.26 ± 0.02	12.25 ± 0.05
λ (nm)	metHb pH 7.4	cod	human
483	min		6.8 ± 0.06*
500	max		7.22 ± 0.05*
522	max	8.48 ± 0.02	
605	min		2.67 ± 0.05*
609	min	2.54 ± 0.00	
630	max	2.91 ± 0.05	3.35 ± 0.05*
λ (nm)	metHb pH 6.5	cod	human
485	min		6.74 ± 0.04*
502	max		7.2 ± 0.04*
522	max	8.61 ± 0.04	
602	min		2.33 ± 0.06*
609	min	2.32 ± 0.01	
630	max	2.76 ± 0.02	
632	max		3.25 ± 0.06*

[a] The methemoglobin (metHb) obtained from the cod and human haemolysate was assumed to be a pure 100% spectrum. The principal minima and maxima millimolar absorptivities (L mmol^{-1} cm^{-1}) (mean ± SEM) in the spectral (λ) region of 450−700 nm are presented. An asterisk (*) indicates significant difference (P value < 0.05) between the cod and human spectra.

the absorptivities of fish and mammalian Hb derivatives as well as absorptivities between pH 7.4 and 6.5 within the species was assessed by a two-tailed Student t test for unpaired samples using SPSS 16.0 for Windows. A difference was considered significant when the P value was below 0.05 (P < 0.05).

The data shown for each Hb derivative at each pH are the mean of three spectra and scaled according to a model ($\varepsilon = A(cl)^{-1}$), made by Steinke and Shepherd,[32] where ε is the 0.25 mM (mM) absorptivity (L mmol^{-1} cm^{-1}), A is the absorbance, c is the concentration expressed in 0.25 mmol L^{-1}, and l is the path length (cm). The mass of 0.25 mmol of Hb is 16.13 g.

The concentration of each individual Hb derivative was determined by using multiple linear regressions with Origin 8.5 for Windows (OriginLab Corp., Northampton, MA). The millimolar absorptivity (mM abs), at any wavelength, is the sum of the contribution of each individual Hb derivative multiplied by an unknown percentage (X). According to this, the formula is

$$\varepsilon_{Hb} = [\varepsilon_{OHb}X\%] + [\varepsilon_{COHb}X\%] + [\varepsilon_{HHb}X\%] + [\varepsilon_{metHb}X\% \text{ at pH } 7.4] + [\varepsilon_{metHb}X\% \text{ at pH } 6.5]$$

In this procedure, the ε_{Hb} spectra obtained after tonometry was assigned the dependent variable, and ε_{OHb}%, ε_{COHb}%, ε_{HHb}%, ε_{metHb}% at pH 7.4, and ε_{metHb}% at pH 6.5 were assigned the independent variables. The OHb, HHb, and COHb spectra obtained after tonometry at pH 7.4, combined with the metHb at both pH 7.4 and 6.5, were used as reference spectra in the multiple linear least-squares curve-fitting procedure. The output of this procedure was the percentage values of the independent variables, which minimize the sum of squares of differences between the observed spectrum and the calculated fit. A 100% spectrum of OHb, COHb, and HHb was calculated from the predicted Hb spectra by setting the accompanying variables to zero.

RESULTS

The fish used were of commercial size and of good biological condition. The muscle pH immediately after death was measured as 7.4 ± 0.1. After 24 h of ice storage, the muscle pH had declined significantly (P < 0.05) to pH 6.4 ± 0.1, and at 7 days postmortem the muscle pH was measured as 6.3 ± 0.1.

The metHb obtained from the cod and human hemolysate, after the addition of potassium ferricyanide, was assumed to be a 100% spectrum. However, the oxyHb, COHb, and HHb spectra obtained after tonometry were not 100% of these derivatives, but rather a mixture of derivatives. Therefore, a spectral deconvolution was used to predict the exact mixture of each individual Hb (OHb, COHb, HHb, and metHb) spectra in the hemolysate after tonometry (Table 1). The coefficient of determination (R^2) between measured Hb spectra and predicted Hb spectra was high, and the prediction error given by the root-mean-square error of prediction (RMSEP) was low, both for the cod and for the human Hb spectra.

Different pH levels induced alterations in mixtures of Hb derivatives after tonometry, and the cod hemolysate was most influenced by changes in pH (Table 1). At pH 7.4, both human and cod hemolysates were almost fully oxygenated when exposed to oxygen during tonometry. However, at pH 6.5, it was mainly the cod Hb that was deoxygenated, and the concentration of HHb and metHb increased. Another pH-induced effect was observed in the predicted oxygenated cod Hb spectrum (Figure 1). It showed slightly lower absorbance peaks at 540 and 575 nm at lower pH. This was not observed with regard to oxygenated human Hb. The main pH-induced difference in human Hb was observed in the metHb spectrum. It showed a pH-induced shift in the spectral band of mammalian metHb (Table 2) at 483−485, 500−502, 602−605, and 630−632 nm. Cod metHb did not show a similar shift. Compared to human metHb, cod metHb was less influenced by changes in pH (Figure 2). No pH-related differences were observed in the cod COHb and HHb spectra or in the human OHb, COHb, and HHb spectra.

Figure 3 shows the Atlantic cod and human Hb absorption spectra of the four OHb, COHb, HHb, and metHb after spectral deconvolution, in the spectral range of 450−700 nm. In Table 2 the absorptivities of the four Hb derivatives of Atlantic cod and

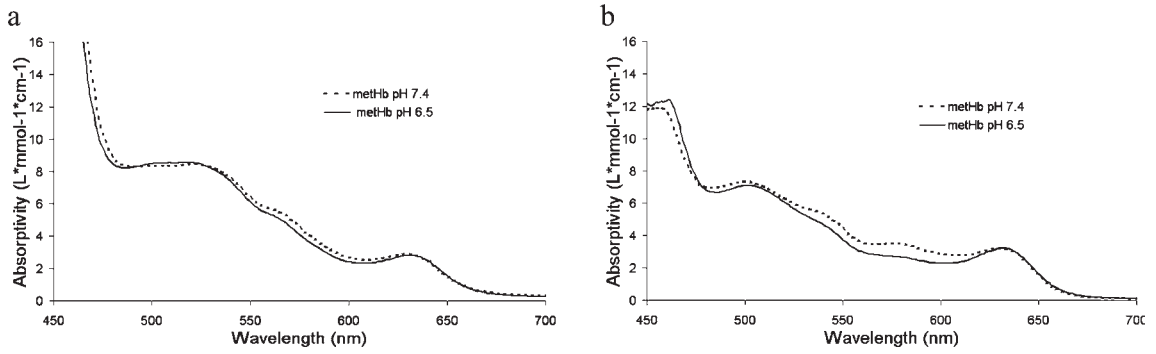

Figure 2. Absorption curves of Atlantic cod (a) and human (b) methemoglobin (metHb) at pH 7.4 and 6.5. The millimolar absorptivities (L mmol^{-1} cm^{-1}) of hemoglobin are presented in the spectral region of 450−700 nm.

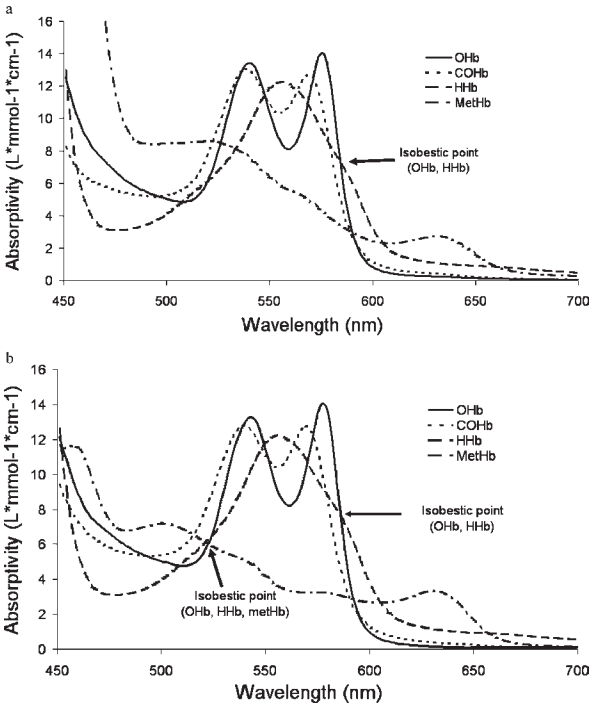

Figure 3. Absorption curves of the four common hemoglobin derivatives of (a) Atlantic cod and (b) human origin. The millimolar absorptivity (L mmol^{-1} cm^{-1}) spectra of 100% oxyhemoglobin (OHb), carboxyhemoglobin (COHb), and deoxyhemoglobin (HHb) were determined by spectral deconvolution and multiple linear least-squares curve fitting procedures. The methemoglobin (metHb) was prepared by adding potassium ferricyanide to the hemolysate and was therefore assumed to be a pure 100% spectrum.

human Hb at the principal light (wavelengths) absorption maxima and minima are presented. Most of the differences in the spectra between human and cod OHb and metHb were statistically significant ($P < 0.05$). For human Hb, a triplicate isosbestic point in the spectra of OHb, HHb, and metHb at 523 nm was found (Figure 3). This point was pH independent as there was found no significant (P value = 0.328) difference in millimolar absorptivities (mM abs) between predicted spectra (mM abs = 6.05 ± 0.05) and the reference spectra obtained at pH 7.4 (mM abs = 5.96 ± 0.06) and pH 6.5 (mM abs = 5.90 ± 0.06). This triplicate isosbestic point was not observed in the absorption spectra of cod Hb. However, the fish metHb spectrum crosses the HHb spectrum at 533 nm (mM abs = 8.15 ± 0.05) and OHb spectrum at 526 nm (mM abs = 8.52 ± 0.04). At 583 nm an isosbestic point between fish OHb and HHb was found. This point is also pH independent (P value = 0.084) at pH 7.4 (mM abs = 7.58 ± 0.06) and at pH 6.5 (mM abs = 7.40 ± 0.05), and the predicted spectra (mM abs = 7.48 ± 0.05) are shown in Figure 3. A similar isosbestic point between human OHb and HHb spectra was also found. However, this point was found at 586 nm (mM abs = 7.52 ± 0.05), which is at a higher wavelength than the isosbestic point in the cod Hb spectra.

In Figure 4, the differences in the absorption spectra between fish and human Hb are presented. This figure clearly shows that there are differences in the Hb absorption spectra of cod and human origin. It also shows that the largest differences were found in the spectra of OHb and metHb.

DISCUSSION

Muscle pH is an important factor when using spectrophotometric multicomponent analysis to predict Hb content in fish muscle samples because the pH influences the absorptive spectra of Hb and its derivatives. The ultimate muscle pH of the fish was 6.4, which is within the normal range for farmed cod. Most fish are filleted post-rigor (after 2−4 days of ice storage), and it is common that muscle pH in fish rapidly decrease within a few hours after death, reaching the ultimate muscle pH after 24 h.[26,27] However, due to the development of pre-rigor filleting, spectrophotometric multicomponent analysis may also be applied to fish filleted pre-rigor, before muscle pH has decreased. Thus, from a fish-processing point of view, at least two pH levels are relevant for carrying out spectrophotometric multicomponent analysis of blood residuals in fillets. In this study, pH 7.4 and 6.5 were chosen to reflect both live muscle pH, that is, fish processed pre-rigor, and ultimate pH, which is relevant for post-rigor-processed fish.

The shape and the isosbestic points in the Hb spectra prepared from human blood (Figure 3) were close to what others have reported.[18,29] The only difference was a lower absorptivity measured in this experiment compared to other studies.[18,29] This might be due to the difference in methods for quantifying Hb. The absorptivity of cyanmethemoglobin measured at 540 nm has been established as a standard for quantifying Hb in blood. However, this assay involves the use of cyanide compounds, which is considered a safety hazard. Therefore, the use of the heme iron method was adopted. The heme iron method converts all forms of Hb into acid hemeatin, with peak transmission

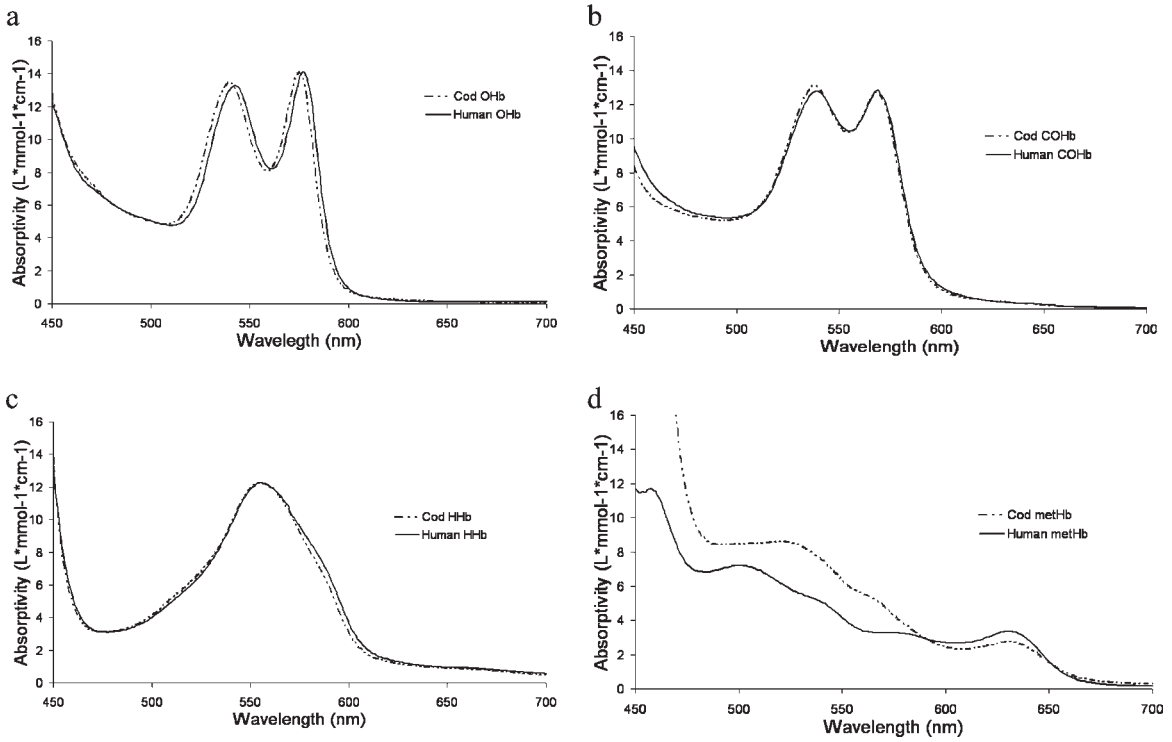

Figure 4. Comparison of the absorption spectra of the four common hemoglobin derivatives of Atlantic cod ($n = 5$) and human ($n = 3$) origin: (a) cod and human OHb absorption spectra; (b) cod and human COHb absorption spectra; (c) cod and human HHb absorption spectra; (d) cod and human metHb absorption spectra at pH 7.4. The millimolar absorptivity (L $mmol^{-1}$ cm^{-1}) spectra of a 100% oxyhemoglobin (OHb), carboxyhemoglobin (COHb), deoxyhemoglobin (HHb), and methemoglobin (metHb) are presented in the spectral region of 450−700 nm.

at 512 and 640 nm.[31] However, this method has the disadvantage of also measuring denatured Hb forms, which cannot be determined by the cyanmethemoglobin method. Consequently, the heme iron method may result in a higher Hb content in samples, which in turn may result in lower millimolar absorptivity. Nevertheless, the heme iron method was well suited for identifying differences between cod and human Hb and pH-induced changes, and differences were found in the Hb spectra between human and cod (Figure 3). The isosbestic point of metHb, HHb, and OHb found at 523 nm, which was found only in the spectra of human Hb, indicates that there are large species-dependent differences in the Hb spectra. Indeed, previous studies have found pH-induced differences in Hb spectra between different fish species[33−35] and even small differences in the Hb spectra between different mammalian species.[18] The differences in the spectra of oxygenated cod hemolysate at pH 6.5 (Figure 1) cannot be explained by interference from HHb or metHb. This is due to the lower absorbance peaks at 540 and 575 nm. However, at lower pH these differences can be explained by protonation of the amino acids in the Hb, which changes the structure of the Hb molecule, but it is also known that error can occur if an unidentified inactive Hb (dyshemoglobin) is present in the hemolysate.[18,30]

Both human and cod hemolysates were almost fully oxygenated at pH 7.4 when exposed to oxygen during tonometry. However, at pH 6.5, especially in cod hemolysate, the concentration of HHb and metHb increases after tonometry (Table 1). At pH 6.5, the cod hemolysate had a substantial amount of HHb (29.5%) and metHb (13.9%). This indicates that the oxygen-carrying capacity was strongly reduced in cod Hb compared to human Hb. This may be explained by the Root effect Hbs in fish blood, which mammalian species lack.[21,23,24] Mammalian Hb, however, is almost fully oxygenated also at pH 6.5. In addition, it can be concluded that cod Hb is more reactive and has a higher rate of autoxidation. A higher rate of autoxidation of fish Hb compared to mammalian Hb has also been found by other researchers.[34−37] Even in freshly drawn fish blood, the content of metHb is high due to spontaneous autoxidation.[38] Therefore, it is recommended that blood samples should be stored on ice prior to analysis to reduce blood cell metabolism and stabilize the autoxidation of Hb.[38,39] During ice storage the rate of autoxidation of Hb in blood equals the kinetic rate of metHb reductase, keeping a stable metHb measurement for some hours.[38] However, the autoxidation of Hb of cold-water fish is found to be 10-fold faster compared to that of warm-water fish.[35] It is therefore very likely that there is a huge difference between fish species, because of their enormous plasticity in the selection of environments.[21]

Carbon monoxide (CO) reversibly binds to Hb, at the same binding site as O_2. The affinity of CO is approximately 240 times greater than that of O_2, and CO will thus bind to Hb preferentially over O_2 when both are present.[40] Due to the strong affinity, the hemolysate was therefore fully saturated with CO at both pH 7.4 and pH 6.5 (Table 1). Fully CO-saturated Hb seems to inhibit autoxidation, compared to fish hemolysate exposed to oxygen at pH 6.5. The only formation of metHb in the COHb solution probably occurred in the Hb solution before tonometry. After tonometry, the Hb solutions were fully saturated with CO at both pH 7.4 and 6.5 and, therefore, protected from autoxidation.

The Root effect Hb in cod blood may also explain why fish Hb is considered a stronger pro-oxidant than mammalian Hb and

may contribute to lipid oxidation in fish muscle during storage. It is known that Hb autoxidation is dependent on pH, the content of HHb, and the presence of O_2. The latter increases the rate of autoxidation.[33,35,37,41−46] In freshly drawn whole blood, both OHb and HHb are the primary chromophores.[47] However, after death, the fish muscle and blood pH will rapidly decline, and this will deoxygenate the pH-sensitive Hb in fish blood. Deoxygenated Hb in the presence of oxygen will initiate a spontaneous autoxidation. Therefore, it is of utmost importance that fish are properly bled and chilled after slaughter. It is also vital that the preslaughter activity is minimized, because an increase in activity before slaughter can influence the amount of Hb in the fish muscle.[8] Proper slaughter, handling, and bleeding are essential and will reduce lipid oxidation during frozen storage.[48] It has been previously reported that the autoxidation rate of fish Hb increases during frozen storage.[38]

It is well-known that there can be large seasonal and individual variation of fish muscle composition.[49] This is important because seasonal variation in muscle glycogen, ATP, and lactate can influence both blood and muscle pH. Also preslaughter activity and temperature, along with red and white blood cell metabolism, can influence blood and muscle pH.[28,50,51] This must be taken into account when fish whole blood or Hb is analyzed. Due to the pH-sensitive Root effect in fish Hb, good laboratory practice, rapid chilling, and good storage conditions of samples are important. Especially the differences in the cod Hb spectra and the speed of the Hb autoxidation compared to human Hb may be crucial for a spectrophotometric multicomponent analysis regarding the presence of blood in fish muscle. In freshly drawn whole blood there will always be a mixture of Hb derivatives, in which OHb and HHb are the major parts. The triplicate isosbestic point at 523 nm in the human Hb spectra is independent of pH and has therefore earlier been used to estimate the total concentration of Hb in solutions containing OHb, HHb, and metHb in any combination.[47,52,53] The concentration of each individual Hb derivative can be calculated from the spectra by using spectral deconvolution. Due to this, there have been some suggestions that this point also could be used to predict residual blood in fish muscle. However, in this experiment such a triplicate isosbestic point between OHb, HHb, and metHb was not found in cod Hb. The reported differences found in the cod Hb can therefore result in large under- or overestimation of Hb content in a muscle sample if the spectra of mammalian Hb are used as a reference, especially if the method used is focusing on only a few specific wavelengths in the visual and near-infrared spectra (400−1100 nm) to predict blood in fish muscle, such as imaging spectroscopy.[7,11] In newly killed white fish muscle, OHb and HHb are the primary chromophores. Therefore, an isosbestic point between only these two Hb derivatives at 583 nm could be used as an alternative to predict the Hb concentration in fish muscle within hours after death. However, fish Hb will rapidly start to oxidize on the surface of a muscle sample, and after some time, metHb will be the primary chromophore. The color of the residual blood will therefore change from dark red to brownish, resulting in changes in the absorption spectra of Hb over time. Time, pH, temperature, and reference method used for calibration are therefore decisive. The assumption that fish blood and mammalian blood perform in the same manner must not be made, because this definitely can contribute to analytical errors in a spectrophotometric analysis. Ideally, a 100% spectrum of all relevant Hb derivatives obtained for each species should be used as a standard for the determination of total Hb content in a sample. A further comparative examination of the function of Hbs within different cold- and warm-water fish species is also needed to characterize possible temperature and pH influences on blood parameters among fish species.

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