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Variation of haemoglobin extinction coefficients can cause errors in the determination of haemoglobin concentration measured by near-infrared spectroscopy

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Abstract

Near-infrared spectroscopy or imaging has been extensively applied to various biomedical applications since it can detect the concentrations of oxyhaemoglobin (HbO_2), deoxyhaemoglobin (Hb) and total haemoglobin (Hb_{total}) from deep tissues. To quantify concentrations of these haemoglobin derivatives, the extinction coefficient values of HbO_2 and Hb have to be employed. However, it was not well recognized among researchers that small differences in extinction coefficients could cause significant errors in quantifying the concentrations of haemoglobin derivatives. In this study, we derived equations to estimate errors of haemoglobin derivatives caused by the variation of haemoglobin extinction coefficients. To prove our error analysis, we performed experiments using liquid-tissue phantoms containing 1% Intralipid in a phosphate-buffered saline solution. The gas intervention of pure oxygen was given in the solution to examine the oxygenation changes in the phantom, and 3 mL of human blood was added twice to show the changes in $[Hb_{total}]$. The error calculation has shown that even a small variation ($0.01\ cm^{-1}\ mM^{-1}$) in extinction coefficients can produce appreciable relative errors in quantification of $\Delta[HbO_2]$, $\Delta[Hb]$ and $\Delta[Hb_{total}]$. We have also observed that the error of $\Delta[Hb_{total}]$ is not always larger than those of $\Delta[HbO_2]$ and $\Delta[Hb]$. This study concludes that we need to be aware of any variation in haemoglobin extinction coefficients, which could result from changes in temperature, and to utilize corresponding animal's haemoglobin extinction coefficients for the animal experiments, in order to obtain more accurate values of $\Delta[HbO_2]$, $\Delta[Hb]$ and $\Delta[Hb_{total}]$ from *in vivo* tissue measurements.

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1. Introduction

In the near-infrared (NIR) region (700–900 nm), light propagating through tissue is mainly absorbed by the haemoglobin in blood, and absorption by other components, such as water and fat, can be negligible. This special characteristic of tissue in the NIR region allows NIR spectroscopy (NIRS) or imaging (NIRI) to be useful in clinical applications since it can detect signals from deep tissue. The NIRS or NIRI technique has been developed for studies of muscles (Chance *et al* 1988, Ferrari *et al* 1992), the brain (Cope and Delpy 1988, Chance *et al* 1998) and tumours (Hull *et al* 1999, Liu *et al* 2000, Ntziachristos and Chance 2001) by many research groups. Most of the algorithms used are based on Beer–Lambert's law, which states that

$$\mu_a = 2.3\varepsilon[C], \tag{1}$$

where μ_a is the absorption coefficient, ε is the extinction coefficient, and $[C]$ is the concentration of absorbing material under measurement, namely, haemoglobin concentration and its derivatives. Equation (1) clearly shows that high accuracy in the determination of $[C]$ necessitates accurate quantification of both μ_a and ε. Researchers have paid great attention to errors caused by the inaccurate determination of absorption coefficients (Fantini *et al* 1995, Pogue and Patterson 1996). In addition, the effects of several factors including the spectral dependence of the differential pathlength factor (DPF) on the accuracy of haemoglobin concentration have been discussed (Strangman *et al* 2003). However, no study has reported that the accuracy of haemoglobin concentration can be significantly affected when there are variations in haemoglobin extinction coefficients.

Tabular forms of haemoglobin extinction coefficients are available mainly from three research groups, namely Zijlstra *et al* (van Kampen and Zijlstra 1965, van Assendelft and Zijlstra 1975, Zijlstra *et al* 1983, 1991, 1994, 2000, Zijlstra and Buursma 1987), Delpy *et al* (Wray *et al* 1988, Cope 1991, Matcher *et al* 1995) and Prahl (1998). Zijlstra's group has extensively reported human haemoglobin extinction coefficients and also reported haemoglobin extinction coefficients from various species (Zijlstra *et al* 2000). However, they showed only a few discrete extinction coefficient values of haemoglobin derivatives in the NIR region while Cope (1991) provided extinction coefficients of human blood in every single unit (nm) of wavelength from 650 nm to beyond 1 μm. Cope (1991) measured human extinction coefficients with their own experiments, compared with other previous reports, and found that the isobestic point of haemoglobin in the NIR region was shifted to 798 $\pm$ 1.5 nm from 800 nm (Horecker 1943), 805 nm (Barlow and Polanyi 1962) and 815 nm (van Assendelft 1970). The haemoglobin extinction coefficient values from Prahl (1998) are only available at the website, not as a published reference. However, his values were adopted in this study to be compared with other groups' extinction coefficients since his extinction coefficients have been widely used by different groups of researchers (Cubeddu *et al* 1999, Heffer *et al* 2004, Torricelli *et al* 2004).

There are several sources which can cause variations in haemoglobin extinction coefficients. Firstly, the extinction coefficient values of haemoglobin derivatives imposed experimental errors when they were obtained. Secondly, the central peaks of laser diodes or LEDs used as light sources in NIRS can deviate from the known centre peaks that the manufacturers originally provided. The wavelength of a laser diode or LED can be shifted by changes of temperature or driving current during experiments. For example, according to the specification of a laser diode from Hitachi (HL7851G 2006), temperature changes from 20 to 30 °C can cause a shift of wavelength from 784 nm to 787 nm, and an operation current change from 135 mA to 165 mA can cause a shift of wavelength from 785 nm to 795 nm.

Thirdly, when we use human haemoglobin extinction coefficients to calculate haemoglobin concentrations from animal experiments, there can be an error in the determination of haemoglobin concentration due to discrepancies of haemoglobin extinction coefficients between humans and animals. According to Zijlstra *et al* (1994), three human haemoglobin extinction coefficients at 750 nm, 775 nm and 800 nm were off by 0.01 (cm^{-1} mM^{-1}) from rat haemoglobin extinction coefficients. In addition, the temperature, pH and sensitivity of detectors can influence the accuracy of determinations in oxyhaemoglobin, [HbO_2], deoxyhaemoglobin, [Hb] and total haemoglobin, [Hb_{total}], concentrations.

Our goal in this paper is to demonstrate that variation of haemoglobin extinction coefficients can cause errors in the determination of haemoglobin concentration. This paper is organized as follows: in section 2, we will estimate the errors of haemoglobin derivative concentrations when there are variations in haemoglobin extinction coefficients induced from several sources. Then the blood-tissue phantom experiments that were used to verify the theoretical error calculation will be described in section 3. The results from error analysis and phantom experiments are presented in section 4, followed by discussion and conclusion in section 5.

2. Error analysis of haemoglobin concentration

2.1. $\Delta[Hb]$, $\Delta[HbO_2]$ and $\Delta[Hb_{total}]$ calculations

With the advantage of using two NIR wavelengths (758 nm and 785 nm in this case), we assume that tissue background absorbance is negligible, and that the main chromophores in human tissue are oxy- and deoxyhaemoglobin molecules. The definition of optical density (OD) is $\mathrm{OD} = \log(I_o/I) = \mu_a L/2.3 = \varepsilon \mathrm{CL}$, where I_o and I are the incident and detected optical intensities in the measurement of a non-scattering medium, respectively, $[C]$ is the concentration of chromophores, in this case haemoglobin derivatives, and L is the optical path length between the source and detector. In the case of a scattering medium, L is not exactly equal to the source–detector separation, d, but rather approximated as $L = d \times \mathrm{DPF}$. The DPF was introduced to take into account light scattering effects in Beer–Lambert's law (Delpy *et al* 1988). With the DPF added into L, we can obtain modified Beer–Lambert's law, which permits us to employ a simplified approach for the calculation of changes in [Hb], [HbO_2] and [Hb_{total}] by obtaining optical density changes from the NIRS measurement, as described previously (Liu *et al* 2000).

Given the above assumption and knowledge, changes in optical density (ΔOD) at two wavelengths can be associated with the changes of [HbO_2] and [Hb] ($\triangle$[HbO_2] and Δ[Hb]) by

$$\begin{pmatrix} \Delta\mathrm{OD}^{\lambda 1} \\ \Delta\mathrm{OD}^{\lambda 2} \end{pmatrix} = \begin{pmatrix} \varepsilon_{\mathrm{Hb}}^{\lambda 1} & \varepsilon_{\mathrm{HbO_2}}^{\lambda 1} \\ \varepsilon_{\mathrm{Hb}}^{\lambda 2} & \varepsilon_{\mathrm{HbO_2}}^{\lambda 2} \end{pmatrix} \begin{pmatrix} \Delta[\mathrm{Hb}] \\ \Delta[\mathrm{HbO_2}] \end{pmatrix} L = \begin{pmatrix} \varepsilon_{\mathrm{Hb}}^{\lambda 1} & \varepsilon_{\mathrm{HbO_2}}^{\lambda 1} \\ \varepsilon_{\mathrm{Hb}}^{\lambda 2} & \varepsilon_{\mathrm{HbO_2}}^{\lambda 2} \end{pmatrix} \begin{pmatrix} \Delta[\mathrm{Hb}] \\ \Delta[\mathrm{HbO_2}] \end{pmatrix} d \cdot \mathrm{DPF}, \tag{2}$$

where $\varepsilon_{\mathrm{Hb}}^{\lambda 1}$, $\varepsilon_{\mathrm{Hb}}^{\lambda 2}$, $\varepsilon_{\mathrm{HbO_2}}^{\lambda 1}$ and $\varepsilon_{\mathrm{HbO_2}}^{\lambda 2}$ are extinction coefficients of deoxygenated and oxygenated haemoglobin at wavelengths of $\lambda 1$ and $\lambda 2$ nm. Then the changes of [Hb] and [HbO_2] can be obtained by the following equation:

$$\begin{pmatrix} \Delta[\mathrm{Hb}] \\ \Delta[\mathrm{HbO_2}] \end{pmatrix} = \frac{1}{d \cdot \mathrm{DPF}} \begin{pmatrix} \varepsilon_{\mathrm{Hb}}^{\lambda 1} & \varepsilon_{\mathrm{HbO_2}}^{\lambda 1} \\ \varepsilon_{\mathrm{Hb}}^{\lambda 2} & \varepsilon_{\mathrm{HbO_2}}^{\lambda 2} \end{pmatrix}^{-1} \begin{pmatrix} \Delta\mathrm{OD}^{\lambda 1} \\ \Delta\mathrm{OD}^{\lambda 2} \end{pmatrix}. \tag{3}$$

The quantity of $\Delta\mathrm{OD}^{\lambda}$ at λ can be expressed by the changes in the detected light intensity, i.e., $\Delta\mathrm{OD}^{\lambda} = \mathrm{OD}_T^{\lambda} - \mathrm{OD}_B^{\lambda} = \log(I_o/I_T)^{\lambda} - \log(I_o/I_B)^{\lambda} = \log(I_B/I_T)^{\lambda}$, where I_B and I_T are

measured optical intensities under baseline and transient conditions, respectively. Then, the changes in optical density at two wavelengths can be replaced with the changes of detected light intensities at two wavelengths, and equation (3) becomes equation (4).

$$\begin{pmatrix} \Delta[\mathrm{Hb}] \\ \Delta[\mathrm{HbO_2}] \end{pmatrix} = \frac{1}{d \cdot \mathrm{DPF}} \cdot \frac{1}{\varepsilon_{\mathrm{Hb}}^{\lambda 2}\varepsilon_{\mathrm{HbO_2}}^{\lambda 1} - \varepsilon_{\mathrm{Hb}}^{\lambda 1}\varepsilon_{\mathrm{HbO_2}}^{\lambda 2}} \begin{pmatrix} -\varepsilon_{\mathrm{HbO_2}}^{\lambda 2} & \varepsilon_{\mathrm{HbO_2}}^{\lambda 1} \\ \varepsilon_{\mathrm{Hb}}^{\lambda 2} & -\varepsilon_{\mathrm{Hb}}^{\lambda 1} \end{pmatrix} \begin{pmatrix} \log\left(\frac{I_B}{I_T}\right)^{\lambda 1} \\ \log\left(\frac{I_B}{I_T}\right)^{\lambda 2} \end{pmatrix}. \tag{4}$$

Furthermore, the change in total haemoglobin concentration is the sum of the changes in [Hb] and [HbO_2] and expressed as equation (5):

$$\Delta[\mathrm{Hb_{total}}] = \Delta[\mathrm{Hb}] + \Delta[\mathrm{HbO_2}]. \tag{5}$$

As a simplified condition, L ($= d \times$ DPF) is assumed to be a constant within the range of the wavelengths that we use because our focus in this paper is on the errors of Δ[Hb], Δ[HbO_2] and Δ[Hb_{total}] caused by variations of only haemoglobin extinction coefficients. In principle, the unit for Δ[Hb], Δ[HbO_2] and Δ[Hb_{total}] is mM. Since L, and so DPF, is a constant over the NIR range in our study, we basically include DPF into the unit as a scaling factor, which will not affect the error analysis induced by variations of extinction coefficients. Thus, in the succeeding sections of the paper, the unit for Δ[Hb], Δ[HbO_2] and Δ[Hb_{total}] will be mM/DPF.

2.2. Error calculation of Δ[Hb], Δ[HbO_2] and Δ[Hb_{total}] caused by variation of haemoglobin extinction coefficients

Since $\varepsilon_{\mathrm{Hb}}^{\lambda 1}$, $\varepsilon_{\mathrm{Hb}}^{\lambda 2}$, $\varepsilon_{\mathrm{HbO_2}}^{\lambda 1}$ and $\varepsilon_{\mathrm{HbO_2}}^{\lambda 2}$ are independent from each other, the errors in Δ[Hb], Δ[HbO_2] and Δ[Hb_{total}] caused by the variations in all of $\varepsilon_{\mathrm{Hb}}^{\lambda 1}$, $\varepsilon_{\mathrm{Hb}}^{\lambda 2}$, $\varepsilon_{\mathrm{HbO_2}}^{\lambda 1}$ and $\varepsilon_{\mathrm{HbO_2}}^{\lambda 2}$can be estimated using the following error propagation principle (Taylor 1997):

$$\Delta x = \pm \left(\sum_{i=1}^{4} \left(\frac{\partial x}{\partial \varepsilon_i} \Delta \varepsilon_i \right)^2 \right)^{\frac{1}{2}}, \tag{6}$$

where x is Δ[Hb] or Δ[HbO_2] or Δ[Hb_{total}], and $\Delta\varepsilon_1$, $\Delta\varepsilon_2$, $\Delta\varepsilon_3$ and $\Delta\varepsilon_4$ are the uncertainties in $\varepsilon_{\mathrm{Hb}}^{\lambda 1}$, $\varepsilon_{\mathrm{Hb}}^{\lambda 2}$, $\varepsilon_{\mathrm{HbO_2}}^{\lambda 1}$ and $\varepsilon_{\mathrm{HbO_2}}^{\lambda 2}$, respectively. To facilitate the computation, let us define the following parameters:

$$C1 = \log\left(\frac{I_B}{I_T}\right)^{\lambda 1}, \qquad C2 = \log\left(\frac{I_B}{I_T}\right)^{\lambda 2}, \tag{7}$$

and

$$D = \varepsilon_{\mathrm{Hb}}^{\lambda 2}\varepsilon_{\mathrm{HbO_2}}^{\lambda 1} - \varepsilon_{\mathrm{Hb}}^{\lambda 1}\varepsilon_{\mathrm{HbO_2}}^{\lambda 2}. \tag{8}$$

After substituting equations (7) and (8) into equations (4) and (5), Δ[Hb], Δ[HbO_2] and Δ[Hb_{total}] can be expressed as

$$\Delta[\mathrm{Hb}] = \frac{1}{d} \frac{\left(-\varepsilon_{\mathrm{HbO_2}}^{\lambda 2} \cdot C1 + \varepsilon_{\mathrm{HbO_2}}^{\lambda 1} \cdot C2\right)}{D}, \tag{9}$$

$$\Delta[\mathrm{HbO_2}] = \frac{1}{d} \frac{\left(\varepsilon_{\mathrm{Hb}}^{\lambda 2} \cdot C1 - \varepsilon_{\mathrm{Hb}}^{\lambda 1} \cdot C2\right)}{D}, \tag{10}$$

$$\Delta[\mathrm{Hb_{total}}] = \frac{1}{d} \frac{\left[\left(\varepsilon_{\mathrm{Hb}}^{\lambda 2} - \varepsilon_{\mathrm{HbO_2}}^{\lambda 2}\right) \cdot C1 + \left(\varepsilon_{\mathrm{HbO_2}}^{\lambda 1} - \varepsilon_{\mathrm{Hb}}^{\lambda 1}\right) \cdot C2\right]}{D}. \tag{11}$$

Note that the factor of DPF has been included in the unit as mM/DPF.

By obtaining the derivatives of equations (9)–(11) and replacing them in equation (6), errors of $\Delta[\mathrm{Hb}]$, $\Delta[\mathrm{HbO_2}]$ and $\Delta[\mathrm{Hb_{total}}]$ caused by $\Delta\varepsilon^{\lambda 1}_{\mathrm{Hb}}$, $\Delta\varepsilon^{\lambda 2}_{\mathrm{Hb}}$, $\Delta\varepsilon^{\lambda 1}_{\mathrm{HbO_2}}$ and $\Delta\varepsilon^{\lambda 2}_{\mathrm{HbO_2}}$ can be quantified as follows:

$$\begin{aligned}\Delta\{\Delta[\mathrm{Hb}]\} = \pm \Bigg\{ & \left(\Delta\varepsilon^{\lambda 1}_{\mathrm{Hb}} \cdot \frac{\varepsilon^{\lambda 2}_{\mathrm{HbO_2}}}{d} \frac{\left(-\varepsilon^{\lambda 2}_{\mathrm{HbO_2}} \cdot C1 + \varepsilon^{\lambda 1}_{\mathrm{HbO_2}} \cdot C2\right)}{D^2} \right)^2 \\ & + \left(\Delta\varepsilon^{\lambda 2}_{\mathrm{Hb}} \cdot \frac{-\varepsilon^{\lambda 1}_{\mathrm{HbO_2}}}{d} \frac{\left(-\varepsilon^{\lambda 2}_{\mathrm{HbO_2}} \cdot C1 + \varepsilon^{\lambda 1}_{\mathrm{HbO_2}} \cdot C2\right)}{D^2} \right)^2 \\ & + \left(\Delta\varepsilon^{\lambda 1}_{\mathrm{HbO_2}} \cdot \frac{\varepsilon^{\lambda 2}_{\mathrm{HbO_2}}}{d} \frac{\left(\varepsilon^{\lambda 2}_{\mathrm{Hb}} \cdot C1 - \varepsilon^{\lambda 1}_{\mathrm{Hb}} \cdot C2\right)}{D^2} \right)^2 \\ & + \left(\Delta\varepsilon^{\lambda 2}_{\mathrm{HbO_2}} \cdot \frac{-\varepsilon^{\lambda 1}_{\mathrm{HbO_2}}}{d} \frac{\left(\varepsilon^{\lambda 2}_{\mathrm{Hb}} \cdot C1 - \varepsilon^{\lambda 1}_{\mathrm{Hb}} \cdot C2\right)}{D^2} \right)^2 \Bigg\}^{1/2} \end{aligned} \tag{12}$$

$$\begin{aligned}\Delta\{\Delta[\mathrm{HbO_2}]\} = \pm \Bigg\{ & \left(\Delta\varepsilon^{\lambda 1}_{\mathrm{Hb}} \cdot \frac{\varepsilon^{\lambda 2}_{\mathrm{Hb}}}{d} \frac{\left(\varepsilon^{\lambda 2}_{\mathrm{HbO_2}} \cdot C1 - \varepsilon^{\lambda 1}_{\mathrm{HbO_2}} \cdot C2\right)}{D^2} \right)^2 \\ & + \left(\Delta\varepsilon^{\lambda 2}_{\mathrm{Hb}} \cdot \frac{-\varepsilon^{\lambda 1}_{\mathrm{Hb}}}{d} \frac{\left(\varepsilon^{\lambda 2}_{\mathrm{HbO_2}} \cdot C1 - \varepsilon^{\lambda 1}_{\mathrm{HbO_2}} \cdot C2\right)}{D^2} \right)^2 \\ & + \left(\Delta\varepsilon^{\lambda 1}_{\mathrm{HbO_2}} \cdot \frac{\varepsilon^{\lambda 2}_{\mathrm{Hb}}}{d} \frac{\left(-\varepsilon^{\lambda 2}_{\mathrm{Hb}} \cdot C1 + \varepsilon^{\lambda 1}_{\mathrm{Hb}} \cdot C2\right)}{D^2} \right)^2 \\ & + \left(\Delta\varepsilon^{\lambda 2}_{\mathrm{HbO_2}} \cdot \frac{-\varepsilon^{\lambda 1}_{\mathrm{Hb}}}{d} \frac{\left(-\varepsilon^{\lambda 2}_{\mathrm{Hb}} \cdot C1 + \varepsilon^{\lambda 1}_{\mathrm{Hb}} \cdot C2\right)}{D^2} \right)^2 \Bigg\}^{1/2} \end{aligned} \tag{13}$$

$$\begin{aligned}\Delta\{\Delta[\mathrm{Hb_{total}}]\} = \pm \Bigg\{ & \left(\Delta\varepsilon^{\lambda 1}_{\mathrm{Hb}} \cdot \frac{\left(\varepsilon^{\lambda 2}_{\mathrm{Hb}} - \varepsilon^{\lambda 2}_{\mathrm{HbO_2}}\right)}{d} \cdot \frac{\left(\varepsilon^{\lambda 2}_{\mathrm{HbO_2}} \cdot C1 - \varepsilon^{\lambda 1}_{\mathrm{HbO_2}} \cdot C2\right)}{D^2} \right)^2 \\ & + \left(\Delta\varepsilon^{\lambda 2}_{\mathrm{Hb}} \cdot \frac{\left(\varepsilon^{\lambda 1}_{\mathrm{HbO_2}} - \varepsilon^{\lambda 1}_{\mathrm{Hb}}\right)}{d} \cdot \frac{\left(\varepsilon^{\lambda 2}_{\mathrm{HbO_2}} \cdot C1 - \varepsilon^{\lambda 1}_{\mathrm{HbO_2}} \cdot C2\right)}{D^2} \right)^2 \\ & + \left(\Delta\varepsilon^{\lambda 1}_{\mathrm{HbO_2}} \cdot \frac{\left(\varepsilon^{\lambda 2}_{\mathrm{HbO2}} - \varepsilon^{\lambda 2}_{\mathrm{Hb}}\right)}{d} \cdot \frac{\left(\varepsilon^{\lambda 2}_{\mathrm{Hb}} \cdot C1 - \varepsilon^{\lambda 1}_{\mathrm{Hb}} \cdot C2\right)}{D^2} \right)^2 \\ & + \left(\Delta\varepsilon^{\lambda 2}_{\mathrm{HbO_2}} \cdot \frac{\left(\varepsilon^{\lambda 1}_{\mathrm{Hb}} - \varepsilon^{\lambda 1}_{\mathrm{HbO_2}}\right)}{d} \cdot \frac{\left(\varepsilon^{\lambda 2}_{\mathrm{Hb}} \cdot C1 - \varepsilon^{\lambda 1}_{\mathrm{Hb}} \cdot C2\right)}{D^2} \right)^2 \Bigg\}^{1/2}. \end{aligned} \tag{14}$$

3. Phantom experiments

3.1. Materials and methods

Blood-tissue phantoms were prepared to investigate the error calculations given above. Two packets of phosphate-buffered saline powder (product no. P-3813, pH 7.4 at 25 °C, SIGMA,

St Louis, MO) were dissolved into 2 L of deionized water to prevent the possible acidification of the solution due to yeasts and to maintain a constant pH level for the phantom. After that, 1% solution of Intralipid was made by adding 105 mL of Intralipid (20% i.v. fat emulsion, Baxter Healthcare Corp., Deerfield, IL) into 2 L of a phosphate-buffered saline solution to have similar optical properties of tissue ($\mu_a = 0.023$ cm^{-1} and $\mu_s' \cong 10$ cm^{-1}). We kneaded 14 g of yeast with 20 mL of deionized water and added them into the phantom solution 5 min before the baseline measurement to avoid optical property changes of the phantom solution during the experiment. After 1 min of baseline measurement, 3 mL of human blood was added twice into the solution during the experiment. During the deoxygenation cycle, the blood was deoxygenated by yeast; during the oxygenation cycle, 100% oxygen gas was introduced into the solution to oxygenate blood. After the blood in the solution was fully oxygenated, bubbling of 100% oxygen was stopped. Another cycle of deoxygenation caused by yeast in the solution then started. Since haemoglobin extinction coefficient values available from the literature were measured at room temperature, we have used a room temperature phantom solution and found small changes in temperature (± 1 °C) during the phantom measurement.

We also conducted a similar blood phantom experiment using horse blood, i.e., 1 L of 1% Intralipid solution with 15 mL of horse blood. The purpose of this phantom experiment was to prove that there could be errors in Δ[Hb], Δ[HbO_2] and Δ[Hb_{total}] calculation if the human extinction coefficients were used, rather than the corresponding ε values from the same species. In this experiment, nitrogen gas was introduced into the solution for the deoxygenation process, and the baseline was measured at the deoxygenation stage. Pure oxygen gas was utilized to induce oxygenation of the blood phantom.

3.2. NIR spectroscopy

A detailed description of our NIRS system can be found in our earlier report (Liu *et al* 2000). Briefly, we used a homodyne, frequency-domain photon migration system to measure the changes of amplitude and phase during the phantom experiments. The light sources were two laser diodes at 758 and 785 nm and modulated at 140 MHz, and the detector was a photomultiplier tube (PMT). The source and detector probes were placed on the side of the container at the same height so that light could pass through the blood tissue phantom in semi-reflectance geometry. The separation of the source and detector was $d = 2$ cm. Figure 1 shows the schematic experimental setup for the blood tissue phantom measurement. After adding yeast, the baseline readings were taken, and 3 mL of human blood was added twice. For oxygenation, pure oxygen gas was bubbled through a plastic tube from the top of the container to oxygenate the solution. Introduction of oxygen gas into the phantom might cause the possible formation of gas bubbles which could affect the scattering properties of the phantom solution. To minimize this potential error, we used a large container (2.5 L) and placed the gas tube opposite to the optical probes location (with a separation > 15 cm) with a moderate oxygen flow rate.

In principle, we should be able to obtain absolute calculations of [HbO_2], [Hb] and blood oxygen saturation (sO_2) since our NIR system could give both phase and amplitude values (Kohl *et al* 1996, Yang *et al* 1997). However, most of our studies have focused on animal tumours (Liu *et al* 2000, Kim *et al* 2003, Gu *et al* 2003), which often have small sizes and large spatial heterogeneity, so it is inaccurate to obtain absolute quantification of haemoglobin concentrations using the conventional diffusion approximation (Fishkin and Gratton 1993). Instead, we have utilized modified Beer–Lambert's law and used only the amplitude of light to calculate Δ[HbO_2], Δ[Hb] and Δ[Hb_{total}] of the tumour caused by gas intervention. This is why we are not using phase readings to perform the error analysis in this study.

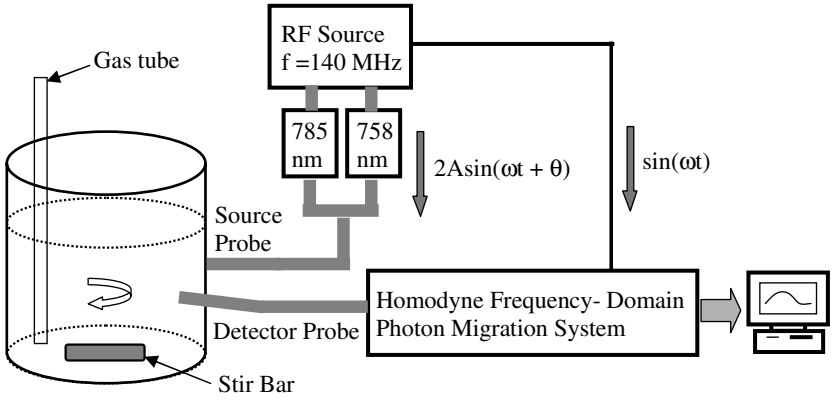

Figure 1. Experimental setup for phantom study using a 1% Intralipid solution with a saline buffer. NIRS probes were placed in semi-reflectance mode, while the gas tube was placed far from the probes to minimize signal artefacts caused by the liquid movement.

Table 1. Haemoglobin extinction coefficients from three different groups.

	ε_{Hb} ($mM^{-1}cm^{-1}$)					ε_{HbO_2} ($mM^{-1}cm^{-1}$)				
Wavelength (nm)	Zijlstra *et al* (1991)	Zijlstra *et al* (1994)	Zijlstra *et al* (2000)	Cope (1991)	Prahl (1998)	Zijlstra *et al* (1991)	Zijlstra *et al* (1994)	Zijlstra *et al* (2000)	Cope (1991)	Prahl (1998)
750	1.56	1.48	1.552	1.5458	1.4052	0.56	0.6	0.592	0.5495	0.518
758		1.416	1.668	1.6820	1.5605		0.6384	0.628	0.5974	0.574
775	1.16	1.28	1.226	1.2481	1.1883	0.68	0.72	0.706	0.7038	0.6832
785		1.104	0.996	0.9975	0.977		0.768	0.756	0.7681	0.7354
800	0.80	0.84	0.86	0.8399	0.7617	0.80	0.84	0.832	0.8653	0.816

For the horse blood phantom experiment, we used a four-channel, continuous-wave, NIRS system which provided only amplitude values of the transmitted light. The light source of this system was from a two-wavelength LED at 730 nm and 850 nm, and four photodiodes were used as detectors. The geometry for the experimental setup was similar to that shown in figure 1 with only a single channel being utilized for the experiment.

4. Results

4.1. Error calculation

Since the wavelengths of the light sources used in our homodyne, frequency-domain NIRS system were 758 and 785 nm, we have obtained haemoglobin extinction coefficient values at these two specific wavelengths from three different groups (table 1). Extinction coefficients given in the left three columns of ε_{Hb} and ε_{HbO_2} in table 1 are from Zijlstra *et al*'s reports (Zijlstra *et al* 1991, 1994, 2000), and those in the fourth and fifth column of ε_{Hb} and ε_{HbO_2} are from Cope (1991) and Prahl (1998), respectively. Since the tabular form of extinction coefficients from Zijlstra *et al* (1994) does not have ε values of Hb and HbO_2 at 758 and 785 nm, the values listed were obtained by a linear interpolation between 750 and 775 nm and between 775 and 800 nm, respectively. In a similar way, ε values of Hb and HbO_2 at 775 and 785 nm from Prahl (1998) and Zijlstra *et al* (2000) were obtained by a linear interpolation between 774 and 776 nm and between 784 and 786 nm, respectively. The haemoglobin

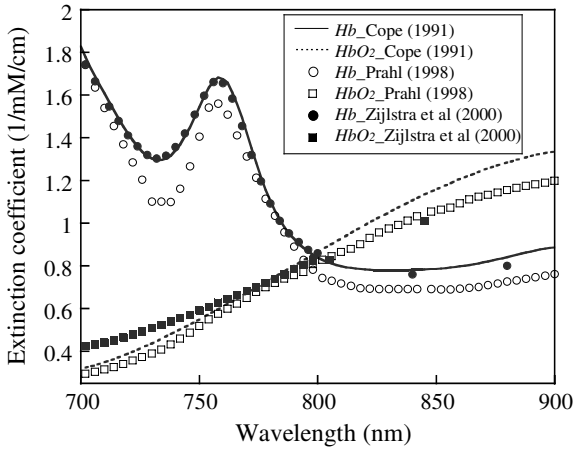

Figure 2. Haemoglobin NIR absorption spectra from Cope (1991), Prahl (1998) and Zijlstra *et al* (2000).

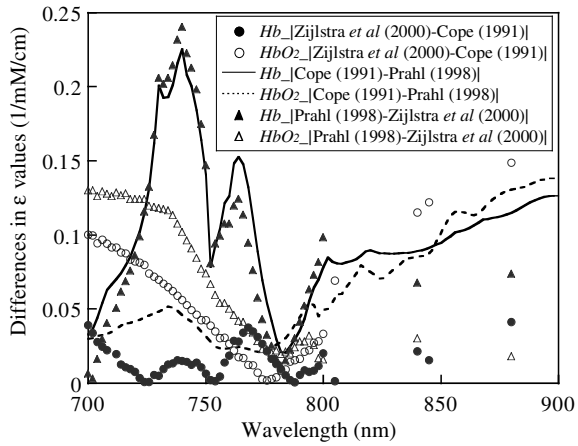

Figure 3. Differences in haemoglobin absorption spectra among Cope (1991), Prahl (1998) and Zijlstra *et al* (2000).

extinction coefficients, ε, from Zijlstra *et al* are 'per equivalent' or 'per haem' values, and thus they were multiplied by 4 to be considered as four haems per haemoglobin so that they can be compared to the ε values given by Cope (1991) and Prahl (1998). To be more complete, the haemoglobin extinction coefficients in the NIR range (700 to 900 nm) from Cope (1991), Prahl (1998), and Zijlstra *et al* (2000) are plotted in figure 2.

As shown in figure 2, the spectra of $\varepsilon_{\mathrm{Hb}}$ and $\varepsilon_{\mathrm{HbO_2}}$ given by the three respective groups are similar but with notable differences from one another. To determine how much deviations in $\Delta[\mathrm{HbO_2}]$, $\Delta[\mathrm{Hb}]$ and $\Delta[\mathrm{Hb_{total}}]$ can result from the discrepancies in haemoglobin extinction coefficients among the groups, we first obtained the differences of haemoglobin ε values among the three groups as shown in table 2. Figure 3 shows the spectral differences of haemoglobin ε values among Cope (1991), Prahl (1998) and Zijlstra *et al* (2000) within the

Table 2. Differences in haemoglobin extinction coefficients among three different groups.

	$\Delta\varepsilon_{Hb}$ ($mM^{-1}cm^{-1}$)				$\Delta\varepsilon_{HbO_2}$ ($mM^{-1}cm^{-1}$)			
Wavelength (nm)	Zijlstra *et al* (1994) versus Zijlstra *et al* (2000)	Zijlstra *et al* (2000) versus Cope (1991)	Zijlstra *et al* (2000) versus Prahl (1998)	Cope (1991) versus Prahl (1998)	Zijlstra *et al* (1994) versus Zijlstra *et al* (2000)	Zijlstra *et al* (2000) versus Cope (1991)	Zijlstra *et al* (2000) versus Prahl (1998)	Cope (1991) versus Prahl (1998)
750	0.0720	0.0062	0.1468	0.1406	0.0080	0.0425	0.074	0.0315
758	0.2520	0.0140	0.1075	0.1215	0.0104	0.0306	0.054	0.0234
775	0.0540	0.0221	0.0377	0.0598	0.0140	0.0022	0.0228	0.0206
785	0.1080	0.0015	0.0190	0.0205	0.0120	0.0121	0.0206	0.0327
800	0.0200	0.0201	0.0983	0.0782	0.0080	0.0333	0.0160	0.0493

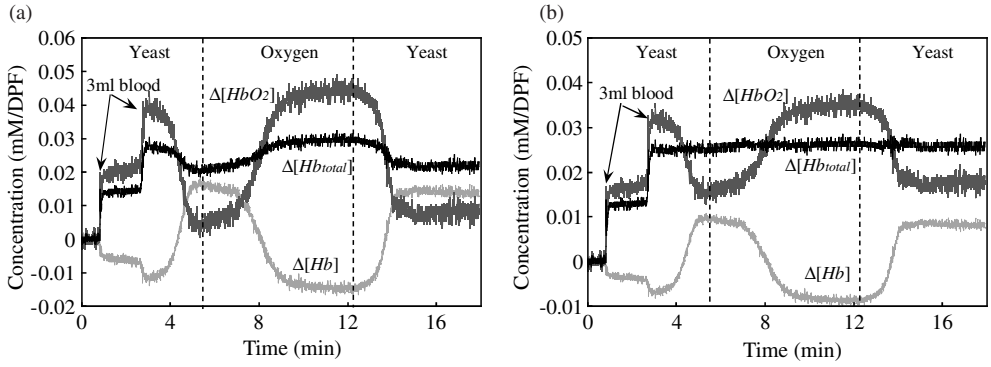

Figure 4. Changes of [Hb], [HbO_2] and [Hb_{total}] taken from a human blood tissue phantom experiment; the respective values were calculated by utilizing haemoglobin extinction coefficients from (a) Zijlstra *et al* (1994) and (b) Zijlstra *et al* (2000).

NIR range (700 nm to 900 nm). Note that there are only a few points available from 800 nm to 900 nm between Zijlstra *et al* (2000) versus Cope (1991) and Zijlstra *et al* (2000) versus Prahl (1998) since Zijlstra *et al* (2000) have published only four ε values of Hb and HbO_2 in this wavelength range.

Once we obtained the differences of haemoglobin extinction coefficients at 758 and 785 nm among the three groups, the values of $\Delta\varepsilon_{Hb}^{\lambda 1}$, $\Delta\varepsilon_{Hb}^{\lambda 2}$, $\Delta\varepsilon_{HbO_2}^{\lambda 1}$ and $\Delta\varepsilon_{HbO_2}^{\lambda 2}$ are available for error calculations using equations (12)–(14) with C1, C2 and D given by equations (7) and (8). Specifically, we utilized a set of amplitudes at $\lambda 1 = 758$ nm and $\lambda 2 = 785$ nm taken during the deoxygenated state (1 min before oxygen intervention) from our tissue phantom experiment (figure 4) with the following readings:

$$\begin{aligned} C1 &= \log\left(\frac{I_b}{I_t}\right)^{\lambda 1} = \log\left(\frac{297}{264}\right)^{758} = 0.0512, \\ C2 &= \log\left(\frac{I_b}{I_t}\right)^{\lambda 2} = \log\left(\frac{148}{134}\right)^{785} = 0.0432. \end{aligned} \tag{15}$$

Substituting equation (15) and the haemoglobin ε values at 758 nm and 785 nm given by Zijlstra *et al* (1994) into equation (9)–(11) leads to values of Δ[Hb], Δ[HbO_2] and Δ[Hb_{total}]:

$$\Delta[\mathrm{Hb}] = 15.33(\mu\mathrm{M/DPF}), \tag{16}$$

$$\Delta[\mathrm{HbO_2}] = 6.06(\mu\mathrm{M/DPF}), \tag{17}$$

$$\Delta[\mathrm{Hb_{total}}] = 21.39(\mu\mathrm{M/DPF}). \tag{18}$$

Based on table 2, we further obtain $\Delta\varepsilon_{Hb}^{758} = 0.252\ \mathrm{mM^{-1}cm^{-1}}$, $\Delta\varepsilon_{Hb}^{785} = 0.108\ \mathrm{mM^{-1}cm^{-1}}$, $\Delta\varepsilon_{HbO_2}^{758} = 0.0104\ \mathrm{mM^{-1}cm^{-1}}$ and $\Delta\varepsilon_{HbO_2}^{785} = 0.012\ \mathrm{mM^{-1}cm^{-1}}$, as the variation or uncertainty of haemoglobin extinction coefficients between Zijlstra *et al* (1994) and (2000). The corresponding relative deviations in Δ[Hb], Δ[HbO_2] and Δ[Hb_{total}] are calculated using equations (12)–(14) with respect to equations (16)–(18):

$$\frac{\Delta\{\Delta[\mathrm{Hb}]\}}{\Delta[\mathrm{Hb}]} = \frac{8.3}{15.33} \times 100\% = 54.1\%, \tag{19}$$

Table 3. The relative errors caused by discrepancies of haemoglobin extinction coefficients.

Case 1: Zijlstra *et al* (1994) versus Zijlstra *et al* (2000)
($\Delta\varepsilon_{Hb}^{758} = 0.252$, $\Delta\varepsilon_{Hb}^{785} = 0.108$, $\Delta\varepsilon_{HbO_2}^{758} = 0.0104$ and $\Delta\varepsilon_{HbO_2}^{785} = 0.012$; all in $mM^{-1}cm^{-1}$)

Concentration (μM/DPF)	A: calculated by using ε from Zijlstra *et al* (1994)	B: deviation (=$\Delta\{\Delta$[Hb derivatives]$\}$)	Relative deviation to A (=100 × B/A) (%)
Δ[Hb]	15.33	8.32	54.3
$\Delta[HbO_2]$	6.06	12.72	209.9
$\Delta[Hb_{total}]$	21.39	6.07	28.4

Case 2: Zijlstra *et al* (2000) versus Cope (1991)
($\Delta\varepsilon_{Hb}^{758} = 0.014$, $\Delta\varepsilon_{Hb}^{785} = 0.0015$, $\Delta\varepsilon_{HbO_2}^{758} = 0.0306$ and $\Delta\varepsilon_{HbO_2}^{785} = 0.0121$; all in $mM^{-1}cm^{-1}$)

Concentration (μM/DPF)	A: calculated by using ε from Zijlstra *et al* (2000)	B: deviation (= $\Delta\{\Delta$[Hb derivatives]$\}$)	Relative deviation to A (=100× B/A) (%)
Δ[Hb]	9.10	1.26	13.8
$\Delta[HbO_2]$	16.55	0.97	5.9
$\Delta[Hb_{total}]$	25.65	1.93	7.5

Case 3: Cope (1991) versus Prahl (1998)
($\Delta\varepsilon_{Hb}^{758} = 0.1215$, $\Delta\varepsilon_{Hb}^{785} = 0.0205$, $\Delta\varepsilon_{HbO_2}^{758} = 0.0234$, $\Delta\varepsilon_{HbO_2}^{785} = 0.0327$; all in $mM^{-1}cm^{-1}$)

Concentration (μM/DPF)	A: calculated by using ε from Cope (1991)	B: deviation (= $\Delta\{\Delta$[Hb derivatives]$\}$)	Relative deviation to A (=100× B/A) (%)
Δ[Hb]	9.70	2.23	23.0
$\Delta[HbO_2]$	15.49	2.20	14.2
$\Delta[Hb_{total}]$	25.20	4.55	18.1

Case 4: Prahl (1998) versus Zijlstra *et al* (2000)
($\Delta\varepsilon_{Hb}^{758} = 0.1075$, $\Delta\varepsilon_{Hb}^{785} = 0.019$, $\Delta\varepsilon_{HbO_2}^{758} = 0.054$ and $\Delta\varepsilon_{HbO_2}^{785} = 0.0206$; all in $mM^{-1}cm^{-1}$)

Concentration (μM/DPF)	A: calculated by using ε from Prahl (1998)	B: deviation (= $\Delta\{\Delta$[Hb derivatives]$\}$)	Relative deviation to A (=100× B/A) (%)
Δ[Hb]	10.95	2.48	22.6
$\Delta[HbO_2]$	14.80	2.31	15.6
$\Delta[Hb_{total}]$	25.75	3.31	12.9

$$\frac{\Delta\{\Delta[HbO_2]\}}{\Delta[HbO_2]} = \frac{12.7}{6.06} \times 100\% = 209.6\%, \tag{20}$$

$$\frac{\Delta\{\Delta[Hb_{total}]\}}{\Delta[Hb_{total}]} = \frac{6.1}{21.39} \times 100\% = 28.5\%. \tag{21}$$

To more completely compare the calculated uncertainties of Δ[Hb], $\Delta[HbO_2]$ and $\Delta[Hb_{total}]$ using different groups of haemoglobin ε values, we have calculated relative deviations in four cases with different comparative combinations, as shown in table 3. All of the calculations for Δ[Hb], $\Delta[HbO_2]$ and $\Delta[Hb_{total}]$ in this table were quantified using the amplitude values shown in equation (15), $d = 2$ cm, and haemoglobin extinction coefficient values from Zijlstra *et al* (1994) (case 1), Zijlstra *et al* (2000) (case 2), Cope (1991) (case 3) and Prahl (1998) (case 4). All of the values for $\Delta\{\Delta$[Hb derivatives]$\}$ were calculated using (12)–(14). This table clearly shows that the minimum deviation in calculated haemoglobin derivative

concentrations is obtained using Zijlstra *et al*'s (2000) and Cope's (1991) haemoglobin ε values (case 2), with less than 15% relative deviations. The greatest deviation occurred using the ε values given by Zijlstra *et al* (1994 , 2000).

In our calculations, the extinction coefficients of Hb and HbO_2 at 758 nm and 785 nm given by Zijlstra *et al* (1994) were obtained by a linear interpolation between values at 750 nm, 775 nm and 800 nm. This linear interpolation is obviously the source of the big deviation in ε values in comparison with those from Zijlstra *et al* (2000), and thus the largest deviation in $\Delta[\mathrm{Hb}]$, $\Delta[\mathrm{HbO_2}]$ and $\Delta[\mathrm{Hb_{total}}]$ occurred between the two cases of using the ε values by Zijlstra *et al* (1994, 2000). Another notable point shown in table 3 is that the values of $\Delta\{\Delta[\mathrm{Hb_{total}}]\}$ due to discrepancies in haemoglobin extinction coefficients are not always the largest in comparison with those of $\Delta\{\Delta[\mathrm{Hb}]\}$ and $\Delta\{\Delta[\mathrm{HbO_2}]\}$. This point will be discussed later in section 5.

4.2. Phantom experimental results

Figure 4(a) shows temporal profiles of $\Delta[\mathrm{Hb}]$, $\Delta[\mathrm{HbO_2}]$ and $\Delta[\mathrm{Hb_{total}}]$ measured from the human blood tissue phantom using the haemoglobin extinction coefficients given by Zijlstra *et al* (1994) during deoxygenation (by yeast) and oxygenation of blood (by 100% oxygen gas). The first 1 min was measured as a baseline after adding yeast, and then followed by the addition of 3 mL of human blood. Another 3 mL of blood was added 2 min after the first addition. The blood in the solution quickly became deoxygenated by the oxygen consumption of yeast, and at about 6 min, pure oxygen gas was introduced into the solution to oxygenate the blood. It is seen that blood oxygenation became saturated at 12 min, and the blood became deoxygenated again after the oxygen flow was stopped.

Figure 4(b) shows the temporal changes of $[\mathrm{Hb}]$, $[\mathrm{HbO_2}]$ and $[\mathrm{Hb_{total}}]$ from the same phantom experiment, but $\Delta[\mathrm{Hb}]$, $\Delta[\mathrm{HbO_2}]$ and $\Delta[\mathrm{Hb_{total}}]$ values were calculated using haemoglobin extinction coefficients from Zijlstra *et al* (2000). The trends of $\Delta[\mathrm{Hb}]$ and $\Delta[\mathrm{HbO_2}]$ are the same as those shown in figure 4(a), but the trend of $\Delta[\mathrm{Hb_{total}}]$ in figure 4(b) is better than that in figure 4(a). It is expected that the total haemoglobin concentration should not change during a cycle of blood oxygenation and deoxygenation. The fact that $\Delta[\mathrm{Hb_{total}}]$ alters noticeably in figure 4(a) but remains constant in figure 4(b) during blood oxygenation and deoxygenation demonstrates a better accuracy of the haemoglobin extinction coefficients given from Zijlstra *et al*'s recent work (Zijlstra *et al* 2000).

Since table 3 is obtained using our phantom data (as shown in figure 4), we have calculated percentage errors of $\Delta[\mathrm{HbO_2}]$, $\Delta[\mathrm{Hb}]$ and $\Delta[\mathrm{Hb_{total}}]$ between figures 4(a) and (b) and compared the experiment-based error calculations with the mathematical calculations as given in table 3. As an example, we used the temporal points at 5.1 min from both figures 4(a) and (b), where the values of $\Delta[\mathrm{Hb}]$, $\Delta[\mathrm{HbO_2}]$ and $\Delta[\mathrm{Hb_{total}}]$ are 15.33, 6.06, 21.39 μM/DPF and 9.10, 16.55, 25.65 μM/DPF, respectively. Using these values, we quantified percentage errors of $\Delta[\mathrm{Hb}]$, $\Delta[\mathrm{Hb\ O_2}]$ and $\Delta[\mathrm{Hb_{total}}]$ between figures 4(a) and (b), as demonstrated below:

$$\frac{\Delta\{\Delta[\mathrm{Hb}]\}}{\Delta[\mathrm{Hb}]} = \frac{|9.1 - 15.33|}{15.33} \times 100\% = 40.6\%, \tag{22}$$

$$\frac{\Delta\{\Delta[\mathrm{HbO_2}]\}}{\Delta[\mathrm{HbO_2}]} = \frac{|16.55 - 6.06|}{6.06} \times 100\% = 173.1\%, \tag{23}$$

$$\frac{\Delta\{\Delta[\mathrm{Hb_{total}}]\}}{\Delta[\mathrm{Hb_{total}}]} = \frac{|25.65 - 21.39|}{21.39} \times 100\% = 19.9\%. \tag{24}$$

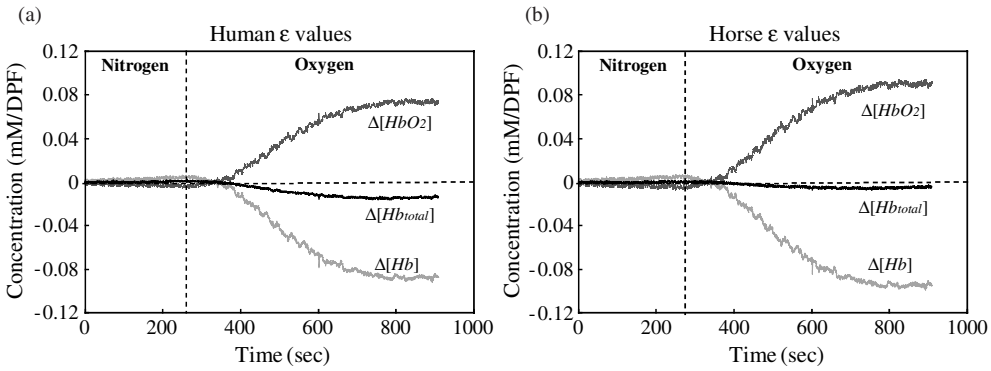

Figure 5. Changes of [Hb], [HbO_2] and [Hb_{total}] taken from a tissue phantom experiment with horse blood; the respective values were calculated by utilizing (a) human haemoglobin extinction coefficients from Cope (1991) and (b) horse haemoglobin extinction coefficients from Zijlstra *et al* (2000).

These experiment-based error values are somewhat smaller than the mathematically based calculations (given in case 1 in table 3), but still in the same order of error magnitude, providing consistent quantification from the experiments to verify the mathematical calculations. Thus, as both mathematical calculations and phantom results have proven, the interpolation approach using the ε values from Zijlstra *et al*'s earlier publication (Zijlstra *et al* 1994) had notable errors, resulting in the required empirical calibrations (Kim *et al* 2003). Moreover, this set of figures exhibits the importance of correct haemoglobin ε values for the accurate determination of [Hb], [HbO_2] and [Hb_{total}].

Figure 5 shows the temporal profiles of Δ[Hb], Δ[HbO_2] and Δ[Hb_{total}] of the phantom experiment with horse blood using either human ε values from Cope (1991) (figure 5(a)) or horse ε values from Zijlstra *et al* (2000) (figure 5(b)). Around 4 min after the baseline, pure oxygen gas was introduced into the solution to oxygenate the blood. These two figures illustrate that the trends of Δ[Hb] and Δ[HbO_2] are similar between the results processed with human and horse ε values. However, the changes of [Hb_{total}] obtained by using human ε values showed a decrease during oxygenation, while the Δ[Hb_{total}] obtained by using horse ε values was maintained nearly constant with respect to the baseline, as expected. This set of data proves the importance of correct ε values in order to obtain accurate calculations for Δ[Hb], Δ[HbO_2] and Δ[Hb_{total}].

5. Discussion and conclusion

To quantify concentrations of haemoglobin derivatives, the extinction coefficient values of HbO_2 and Hb have to be employed. However, few reports have shown how small deviation in extinction coefficients could cause errors in quantifying the concentrations of haemoglobin derivatives. In this study, we derived equations to estimate deviations or errors of haemoglobin derivative concentrations caused by variation of haemoglobin extinction coefficients. To support our error analysis, we conducted experiments using liquid-tissue phantoms containing 1% Intralipid in phosphate-buffered saline mixed with human or horse blood. The gas intervention of pure oxygen was given in the solution to examine the oxygenation changes in the phantom. The error calculation has shown that even a small variation (0.01 cm^{-1} mM^{-1}) in extinction coefficients can produce significant deviations in quantification of Δ[HbO_2], Δ[Hb]

and $\Delta[\mathrm{Hb_{total}}]$. This study clearly demonstrates that it is important to be aware of any variation in haemoglobin extinction coefficients, which could highly affect the accuracy of $\Delta[\mathrm{HbO_2}]$, $\Delta[\mathrm{Hb}]$ and $\Delta[\mathrm{Hb_{total}}]$ from *in vivo* tissue measurements.

The haemoglobin extinction coefficients have been studied for more than five decades by biochemists or clinical chemists to quantify [Hb] and $[\mathrm{HbO_2}]$ in laboratory measurements. For convenient comparison, representative tabulated values of haemoglobin extinction coefficients from several references (Horecker 1943, Benesch *et al* 1965, van Assendelft 1970, Takatani and Graham 1979, Zijlstra *et al* 1991, 1994, 2000, Wray *et al* 1988, Cope 1991, Matcher *et al* 1995, Prahl 1998) are compiled in tables 4 and 5 with a unit of 0.25 $\mathrm{mM^{-1}cm^{-1}}$ which represents the 'per haem' values. In our previous report (Kim *et al* 2005), we have emphasized that the values of haemoglobin extinction coefficients from Zijlstra *et al*'s group have to be multiplied by a factor of 4 since their haemoglobin ε values are expressed in a haem basis. It was clearly stated in early studies that the presented values of haemoglobin extinction coefficients were derived for haemoglobin per equivalent or per haem (Horecker 1943, Benesh *et al* 1965, van Kampen and Zijlstra 1965), but this information gradually has been ignored in more recent publications (Zijlstra and Buursma 1987, Zijlstra *et al* 1994, Sfareni *et al* 1997). With better understanding of this factor of 4, some of our previous studies can be improved for a better accuracy (Liu *et al* 2000, Kim *et al* 2003, Gu *et al* 2003), while all of the published conclusions remain unaffected.

Although our error analysis focuses on the accuracy for changes in [Hb], $[\mathrm{HbO_2}]$ and $[\mathrm{Hb_{total}}]$, all the mathematical derivations given in equations (6) and (12)–(14) can be readily used in error analysis for absolute calculations of [Hb], $[\mathrm{HbO_2}]$ and $[\mathrm{Hb_{total}}]$. It is known that the expressions for [Hb] and $[\mathrm{HbO_2}]$ can be expressed as (Liu *et al* 1995, Yang *et al* 1997)

$$\begin{pmatrix}[\mathrm{Hb}]\\ [\mathrm{HbO_2}]\end{pmatrix} = \frac{1}{2.3} \times \frac{1}{\varepsilon_{\mathrm{Hb}}^{\lambda 2}\varepsilon_{\mathrm{HbO_2}}^{\lambda 1} - \varepsilon_{\mathrm{Hb}}^{\lambda 1}\varepsilon_{\mathrm{HbO_2}}^{\lambda 2}} \begin{pmatrix} -\varepsilon_{\mathrm{HbO_2}}^{\lambda 2} & \varepsilon_{\mathrm{HbO_2}}^{\lambda 1} \\ \varepsilon_{\mathrm{Hb}}^{\lambda 2} & -\varepsilon_{\mathrm{Hb}}^{\lambda 1} \end{pmatrix} \begin{pmatrix} \mu_a^{\lambda 1} \\ \mu_a^{\lambda 2} \end{pmatrix}. \quad (25)$$

The similarity between equations (4) and (25) warrants the validation of the analysis methodology for [Hb], $[\mathrm{HbO_2}]$ and $[\mathrm{Hb_{total}}]$.

As briefly mentioned in section 1, Fantini *et al* (1995) studied the uncertainties in [Hb], $[\mathrm{HbO_2}]$ and $[\mathrm{Hb_{total}}]$ due to propagation of uncertainties in μ_a and μ_s'. In their report, they have shown an equation for the uncertainty in $[\mathrm{Hb_{total}}]$ caused by the standard deviation in μ_a and μ_s' as follows:

$$\sigma([\mathrm{Hb_{total}}]) = \sqrt{\{\sigma([\mathrm{Hb}])\}^2 + \{\sigma([\mathrm{HbO_2}])\}^2}. \quad (26)$$

This equation basically implies that the variation of $[\mathrm{Hb_{total}}]$ caused by uncertainties in μ_a and μ_s' is always larger than those of [Hb] or $[\mathrm{HbO_2}]$. In our study, however, it is seen that the deviation in $\Delta[\mathrm{Hb_{total}}]$ is not always larger than those of $\Delta[\mathrm{Hb}]$ or $\Delta[\mathrm{HbO_2}]$ (B values in case 1 of table 3 and equations (22)–(24)). The disagreement between our study and Fantini *et al*'s report can be interpreted as follows. Equation (26) would be valid with the assumption that $[\mathrm{Hb_{total}}]$ is a dependent variable and [Hb] and $[\mathrm{HbO_2}]$ are two independent variables. However, with a close inspection on [Hb], $[\mathrm{HbO_2}]$ and $[\mathrm{Hb_{total}}]$, one can realize that the actual independent variables should be haemoglobin extinction coefficients and OD (or μ_a in equation (25)), not [Hb] and $[\mathrm{HbO_2}]$. Therefore, equation (14) should give an accurate estimation of uncertainties in $\Delta[\mathrm{Hb_{total}}]$ induced by the discrepancies in haemoglobin extinction coefficients between the reported data.

Temperature can be another important factor that could affect calculations of $\Delta[\mathrm{Hb}]$, $\Delta[\mathrm{HbO_2}]$ and $\Delta[\mathrm{Hb_{total}}]$ measured by NIRS since temperature alters haemoglobin extinction coefficients. Studies have shown that the optical absorbance spectra of haemoglobin derivatives vary with temperature (Cordone *et al* 1986, Steinke and Shepherd 1992, Sfareni *et al* 1997).

Table 4. The per equivalent extinction coefficients of deoxyhaemoglobin (unit: 0.25 $mM^{-1}cm^{-1}$).

Wavelength (nm)	Horecker (1943)	Benesch *et al* (1965)	van Assendelft (1970), Takatani and Graham (1979)	Wray *et al* (1988)	Cope (1991)	Zijlstra *et al* (1991)	Zijlstra *et al* (1994)	Matcher *et al* (1995)	Prahl (1998)	Zijlstra *et al* (2000)
700					0.4568	0.44	0.45	0.4568	0.44857	0.447
701				0.4495	0.4503			0.4503		
702					0.4444			0.4444	0.43525	0.436
703					0.4387			0.4387		
704					0.4330			0.4330	0.42194	0.426
705			0.497	0.4300	0.4275			0.4275		
706					0.4222			0.4222	0.40862	0.416
707					0.4169			0.4169		
708				0.4118	0.4116			0.4116	0.39588	0.406
709					0.4067			0.4067		
710					0.4018			0.4018	0.38512	
711				0.3948	0.3968			0.3968		
712					0.3919			0.3919	0.37435	0.387
713					0.3870			0.3870		
714					0.3821			0.3821	0.36359	0.379
715			0.407	0.3783	0.3773			0.3773		
716					0.3727			0.3727	0.35283	0.37
717					0.3680			0.3680		
718				0.3625	0.3634			0.3634	0.34207	0.361
719					0.3591			0.3591		
720					0.3549			0.3549	0.33147	0.353
721				0.3480	0.3507			0.3507		
722					0.3469			0.3469	0.32129	0.346
723					0.3433			0.3433		
724					0.3398			0.3398	0.31111	0.34
725			0.4655	0.3358	0.3364			0.3364		
726					0.3338			0.3338	0.30092	0.334
727					0.3312			0.3312		
728				0.3268	0.3287			0.3287	0.2882	0.33
729					0.3270			0.3270		
730					0.3257			0.3257	0.27555	0.327
731	0.31				0.3244			0.3244		
732				0.3215	0.3237			0.3237	0.27555	0.326
733					0.3238			0.3238		
734					0.3238			0.3238	0.27555	0.327
735			0.366	0.3215	0.3240			0.3240		
736					0.3257			0.3257	0.27544	0.329
737					0.3274			0.3274		
738				0.3268	0.3291			0.3291	0.27512	0.333
739					0.3319			0.3319		
740					0.3353			0.3353	0.27897	0.339
741					0.3386			0.3386		
742				0.3373	0.3424			0.3424	0.29041	0.346
743					0.3472			0.3472		
744					0.3520			0.3520	0.30185	0.355
745			0.404	0.3530	0.3567			0.3567		
746					0.3625			0.3625	0.31651	0.366
747					0.3684			0.3684		

Table 4. (Continued.)

Wavelength (nm)	Horecker (1943)	Benesch *et al* (1965)	van Assendelft (1970), Takatani and Graham (1979)	Wray *et al* (1988)	Cope (1991)	Zijlstra *et al* (1991)	Zijlstra *et al* (1994)	Matcher *et al* (1995)	Prahl (1998)	Zijlstra *et al* (2000)
748				0.3725	0.3743			0.3743	0.33331	0.377
749					0.3803			0.3803		
750					0.3864	0.39	0.37	0.3864	0.35131	0.388
751					0.3926			0.3926		
752				0.3935	0.3984			0.3984	0.37883	0.399
753					0.4036			0.4036		
754					0.4086			0.4086	0.38544	0.409
755			0.439	0.4110	0.4137			0.4137		
756					0.4162			0.4162	0.39012	0.415
757					0.4183			0.4183		
758				0.4195	0.4205			0.4205	0.39012	0.417
759					0.4204			0.4204		
760	0.395	0.416			0.4186			0.4186	0.38713	0.414
761					0.4169			0.4169		
762				0.4150	0.4142			0.4142	0.37711	0.407
763					0.4086			0.4086		
764					0.4030			0.4030	0.36489	0.396
765			0.41	0.3975	0.3974			0.3974		
766					0.3896			0.3896	0.35263	0.381
767					0.3815			0.3815		
768				0.3713	0.3734			0.3734	0.34033	0.364
769					0.3647			0.3647		
770					0.3557			0.3557	0.32797	0.347
771					0.3467			0.3467		
772				0.3413	0.3378			0.3378	0.31561	0.33
773					0.3292			0.3292		
774					0.3206			0.3206	0.30325	0.314
775			0.335	0.3125	0.3120	0.29	0.32	0.3120		
776					0.3044			0.3044	0.29089	0.299
777					0.2968			0.2968		
778				0.2873	0.2892			0.2892	0.2787	0.285
779					0.2825			0.2825		
780					0.2763			0.2763	0.26886	0.273
781					0.2701			0.2701		
782				0.2665	0.2643			0.2643	0.25902	0.262
783					0.2593			0.2593		
784					0.2543			0.2543	0.24918	0.253
785			0.26	0.2498	0.2494			0.2494		
786					0.2455			0.2455	0.23934	0.245
787					0.2417			0.2417		
788				0.2370	0.2379			0.2379	0.23045	0.238
789					0.2346			0.2346		
790					0.2316			0.2316	0.2227	0.233
791					0.2286			0.2286		
792				0.2270	0.2258			0.2258	0.21495	0.228
793					0.2235			0.2235		
794					0.2211			0.2211	0.2072	0.223
795			0.241	0.2193	0.2188			0.2188		
796					0.2169			0.2169	0.20074	0.219

Table 4. (Continued.)

Wavelength (nm)	Horecker (1943)	Benesch *et al* (1965)	van Assendelft (1970), Takatani and Graham (1979)	Wray *et al* (1988)	Cope (1991)	Zijlstra *et al* (1991)	Zijlstra *et al* (1994)	Matcher *et al* (1995)	Prahl (1998)	Zijlstra *et al* (2000)
797					0.2150			0.2150		
798				0.2130	0.2131			0.2131	0.19559	0.216
799					0.2115			0.2115		
800					0.2100	0.2	0.21	0.2100	0.19043	0.215
801				0.2080	0.2085			0.2085		
802					0.2071			0.2071	0.18596	
803					0.2059			0.2059		
804					0.2047			0.2047	0.18427	
805			0.224	0.2040	0.2036	0.2		0.2036		0.204
806					0.2028			0.2028	0.18257	
807					0.2019			0.2019		
808				0.2010	0.2010			0.2010	0.18088	
809					0.2003			0.2003		
810					0.1997			0.1997	0.17927	
811				0.1990	0.1990			0.1990		
812					0.1985			0.1985	0.17796	
813					0.1980			0.1980		
814				0.1973	0.1976			0.1976	0.17665	
815			0.22		0.1972			0.1972		
816					0.1969			0.1969	0.17533	
817					0.1965			0.1965		
818				0.1963	0.1962			0.1962	0.17402	
819					0.1960			0.1960		
820					0.1959			0.1959	0.17344	
821				0.1955	0.1957			0.1957		
822					0.1955			0.1955	0.1734	
823					0.1954			0.1954		
824				0.1948	0.1953			0.1953	0.17337	
825			0.208		0.1952			0.1952		
826					0.1952			0.1952	0.17333	
827					0.1951			0.1951		
828				0.1945	0.1951			0.1951	0.1733	
829					0.1951			0.1951		
830					0.1951			0.1951	0.17326	
831				0.1945	0.1951			0.1951		
832					0.1951			0.1951	0.17323	
833					0.1951			0.1951		
834				0.1943	0.1951			0.1951	0.17319	
835			0.205		0.1951			0.1951		
836					0.1951			0.1951	0.17316	
837				0.1943	0.1952			0.1952		
838					0.1952			0.1952	0.17312	
839					0.1953			0.1953		
840	0.179			0.1943	0.1954	0.19		0.1954	0.17309	0.19
841					0.1954			0.1954		
842					0.1955			0.1955	0.17305	
843					0.1956			0.1956		
844				0.1945	0.1957			0.1957	0.17299	
845			0.205		0.1958	0.19		0.1958		0.192

Table 4. (Continued.)

Wavelength (nm)	Horecker (1943)	Benesch *et al* (1965)	van Assendelft (1970), Takatani and Graham (1979)	Wray *et al* (1988)	Cope (1991)	Zijlstra *et al* (1991)	Zijlstra *et al* (1994)	Matcher *et al* (1995)	Prahl (1998)	Zijlstra *et al* (2000)
846					0.1960			0.1960	0.17294	
847				0.1950	0.1961			0.1961		
848					0.1963			0.1963	0.17288	
849					0.1964			0.1964		
850				0.1953	0.1965			0.1965	0.17283	
851					0.1968			0.1968		
852					0.1970			0.1970	0.17277	
853				0.1960	0.1973			0.1973		
854					0.1975			0.1975	0.17272	
855			0.205		0.1978			0.1978		
856					0.1981			0.1981	0.17266	
857				0.1970	0.1984			0.1984		
858					0.1987			0.1987	0.17311	
859					0.1991			0.1991		
860				0.1980	0.1994			0.1994	0.17358	
861					0.1998			0.1998		
862					0.2002			0.2002	0.17405	
863				0.1993	0.2006			0.2006		
864					0.2011			0.2011	0.17451	
865			0.205		0.2016			0.2016		
866				0.2008	0.2021			0.2021	0.17498	
867					0.2026			0.2026		
868					0.2031			0.2031	0.17545	
869				0.2025	0.2036			0.2036		
870					0.2041			0.2041	0.17646	
871					0.2047			0.2047		
872				0.2043	0.2053			0.2053	0.17749	
873					0.2059			0.2059		
874					0.2065			0.2065	0.17852	
875			0.212		0.2071			0.2071		
876				0.2060	0.2078			0.2078	0.17955	
877					0.2084			0.2084		
878					0.2090			0.2090	0.18058	
879				0.2083	0.2097			0.2097		
880					0.2103	0.2		0.2103	0.18161	0.2
881					0.2109			0.2109		
882				0.2103	0.2116			0.2116	0.18246	
883					0.2122			0.2122		
884					0.2129			0.2129	0.1833	
885			0.208	0.2125	0.2136			0.2136		
886					0.2142			0.2142	0.18415	
887					0.2148			0.2148		
888				0.2145	0.2155			0.2155	0.18499	
889					0.2161			0.2161		
890					0.2167			0.2167	0.1859	
891				0.2165	0.2174			0.2174		
892					0.2180			0.2180	0.18681	
893					0.2186			0.2186		
894					0.2191			0.2191	0.18772	

Table 4. (Continued.)

Wavelength (nm)	Horecker (1943)	Benesch *et al* (1965)	van Assendelft (1970), Takatani and Graham (1979)	Wray *et al* (1988)	Cope (1991)	Zijlstra *et al* (1991)	Zijlstra *et al* (1994)	Matcher *et al* (1995)	Prahl (1998)	Zijlstra *et al* (2000)
895			0.221	0.2183	0.2197			0.2197		
896					0.2202			0.2202	0.18863	
897					0.2207			0.2207		
898				0.2200	0.2211			0.2211	0.18954	
899					0.2216			0.2216		
900	0.198				0.2220			0.2220	0.19046	

The extinction coefficients used in this report were determined *in vitro* at ambient temperature (20–24 °C) (Zijlstra *et al* 1991, 1994, 2000, Cope 1991). However, the temperature during *in vivo* measurements from human or animal tissues is often ∼37 °C and is significantly higher than that from *in vitro* measurements.

The results reported by Steinke and Shepherd (1992) showed the effect of temperature changes from 20 to 40 °C on haemoglobin extinction coefficients within 480 nm to 650 nm, excluding the NIR range. Their report indicated that temperature had the most pronounced effect on both deoxyhaemoglobin and oxyhaemoglobin extinction coefficients in the wavelength range of 500–610 nm and also showed that oxyhaemoglobin extinction coefficients are more sensitive to changes in temperature than carboxy- or deoxyhaemoglobin extinction coefficients. In this range of wavelengths changes in extinction coefficients ranged from 0.4 to 2.8 $mM^{-1}cm^{-1}$ when temperature changed from 20 to 40 °C.

The report from Cordone *et al* (1986) showed the effect of temperature on extinction coefficients from 650 to 1350 nm, with temperature changes from 25 to −253 °C. In their report, there was an approximate increase of 0.22 $mM^{-1}cm^{-1}$ in the deoxyhaemoglobin extinction coefficient at 758 nm when the temperature dropped from 25 to −73 °C, which can be approximately estimated as a 0.022 $mM^{-1}cm^{-1}$ increase per 10 °C temperature drop. Sfareni *et al* (1997) reported the changes of NIR absorption spectra of haemoglobin in the temperature range of 20–40 °C. It was reported that the deoxyhaemoglobin ε at 758 nm was increased ∼0.036 $mM^{-1}cm^{-1}$ when the temperature was dropped from 40 to 20 °C, and that the oxyhaemoglobin ε decreased around 0.008 $mM^{-1}cm^{-1}$ with a decrease in temperature. The deoxyhaemoglobin ε at 785 nm was roughly decreased 0.032 $mM^{-1}cm^{-1}$ by a decrease of temperature from 40 to 20 °C, while oxyhaemoglobin ε showed an increase of around 0.004 $mM^{-1}cm^{-1}$.

Here we demonstrate the possible errors of $\Delta[\mathrm{Hb}]$, $\Delta[\mathrm{HbO_2}]$ and $\Delta[\mathrm{Hb_{total}}]$ due to temperature changes from 20 to 40 °C. Using the haemoglobin ε values given by Zijlstra *et al* (2000), we obtain deviations in ε values due to temperature as $\Delta\varepsilon^{758}_{\mathrm{Hb}} = 0.036$, $\Delta\varepsilon^{785}_{\mathrm{Hb}} = 0.032$, $\Delta\varepsilon^{758}_{\mathrm{HbO_2}} = 0.008$ and $\Delta\varepsilon^{785}_{\mathrm{HbO_2}} = 0.004$, all in $\mathrm{cm^{-1}\ mM^{-1}}$. With the same parameters as those used to calculate equations (19)–(21), the relative errors of $\Delta[\mathrm{Hb}]$, $\Delta[\mathrm{HbO_2}]$ and $\Delta[\mathrm{Hb_{total}}]$ induced by temperature changes are quantified as follows:

$$\frac{\Delta\{\Delta[\mathrm{Hb}]\}}{\Delta[\mathrm{Hb}]} = \frac{0.6}{9.10} \times 100\% = 6.6\%, \tag{27}$$

$$\frac{\Delta\{\Delta[\mathrm{HbO_2}]\}}{\Delta[\mathrm{HbO_2}]} = \frac{0.96}{16.55} \times 100\% = 5.8\%, \tag{28}$$

Table 5. The per equivalent extinction coefficients of oxyhaemoglobin (unit: 0.25 $mM^{-1}cm^{-1}$).

Wavelength (nm)	Horecker (1943)	Benesch *et al* (1965)	van Assendelft (1970), Takatani and Graham (1979)	Wray *et al* (1988)	Cope (1991)	Zijlstra *et al* (1991)	Zijlstra *et al* (1994)	Matcher *et al* (1995)	Prahl (1998)	Zijlstra *et al* (2000)
700	0.093				0.0800	0.09	0.1	0.0800	0.0725	0.105
701				0.1053	0.0805			0.0805		
702					0.0811			0.0811	0.0735	0.106
703					0.0817			0.0817		
704					0.0824			0.0824	0.0745	0.106
705			0.075	0.1068	0.0831			0.0831		
706					0.0838			0.0838	0.0757	0.108
707					0.0847			0.0847		
708				0.1088	0.0854			0.0854	0.0771	0.109
709					0.0863			0.0863		
710					0.0871			0.0871	0.0785	0.11
711				0.1110	0.0881			0.0881		
712					0.0891			0.0891	0.0799	0.112
713					0.0901			0.0901		
714					0.0910			0.0910	0.0813	0.113
715			0.082	0.1135	0.0920			0.0920		
716					0.0931			0.0931	0.083	0.115
717					0.0941			0.0941		
718				0.1165	0.0952			0.0952	0.085	0.116
719					0.0964			0.0964		
720					0.0975			0.0975	0.087	0.118
721				0.1195	0.0986			0.0986		
722					0.0997			0.0997	0.089	0.12
723					0.1009			0.1009		
724					0.1021			0.1021	0.091	0.122
725			0.092	0.1225	0.1033			0.1033		
726					0.1045			0.1045	0.0931	0.123
727					0.1058			0.1058		
728				0.1258	0.1070			0.1070	0.0953	0.125
729					0.1083			0.1083		
730					0.1096			0.1096	0.0975	0.127
731					0.1109			0.1109		
732				0.1293	0.1122			0.1122	0.0997	0.129
733					0.1135			0.1135		
734					0.1148			0.1148	0.1019	0.131
735			0.103	0.1330	0.1161			0.1161		
736					0.1175			0.1175	0.1047	0.133
737					0.1188			0.1188		
738				0.1365	0.1203			0.1203	0.1081	0.135
739					0.1216			0.1216		
740					0.1230			0.1230	0.1115	0.137
741					0.1244			0.1244		
742				0.1403	0.1258			0.1258	0.1149	0.139
743					0.1272			0.1272		
744					0.1287			0.1287	0.1183	0.141
745			0.12	0.1440	0.1301			0.1301		
746					0.1315			0.1315	0.1219	0.143
747					0.1329			0.1329		

Table 5. (Continued.)

Wavelength (nm)	Horecker (1943)	Benesch *et al* (1965)	van Assendelft (1970), Takatani and Graham (1979)	Wray *et al* (1988)	Cope (1991)	Zijlstra *et al* (1991)	Zijlstra *et al* (1994)	Matcher *et al* (1995)	Prahl (1998)	Zijlstra *et al* (2000)
748				0.1480	0.1344			0.1344	0.1257	0.145
749					0.1359			0.1359		
750					0.1374	0.14	0.15	0.1374	0.1295	0.148
751					0.1388			0.1388		
752				0.1520	0.1403			0.1403	0.1333	0.15
753					0.1418			0.1418		
754					0.1433			0.1433	0.1371	0.152
755			0.139	0.1560	0.1448			0.1448		
756					0.1464			0.1464	0.1405	0.154
757					0.1478			0.1478		
758				0.1603	0.1493			0.1493	0.1435	0.157
759					0.1508			0.1508		
760		0.135			0.1524			0.1524	0.1465	0.159
761					0.1539			0.1539		
762				0.1645	0.1555			0.1555	0.1495	0.161
763					0.1571			0.1571		
764					0.1586			0.1586	0.1525	0.163
765			0.154	0.1688	0.1602			0.1602		
766					0.1617			0.1617	0.1557	0.166
767					0.1634			0.1634		
768				0.1730	0.1649			0.1649	0.1591	0.168
769					0.1665			0.1665		
770					0.1680			0.1680	0.1625	0.171
771					0.1696			0.1696		
772				0.1775	0.1712			0.1712	0.1659	0.173
773					0.1728			0.1728		
774					0.1743			0.1743	0.1693	0.175
775			0.171	0.1820	0.1759	0.17	0.18	0.1759		
776					0.1775			0.1775	0.1723	0.178
777					0.1791			0.1791		
778				0.1863	0.1807			0.1807	0.1749	0.18
779					0.1824			0.1824		
780					0.1840			0.1840	0.1775	0.183
781					0.1855			0.1855		
782				0.1908	0.1871			0.1871	0.1801	0.185
783					0.1887			0.1887		
784					0.1903			0.1903	0.1827	0.188
785			0.184	0.1953	0.1920			0.1920		
786					0.1936			0.1936	0.185	0.19
787					0.1952			0.1952		
788				0.1998	0.1969			0.1969	0.187	0.193
789					0.1985			0.1985		
790					0.2001			0.2001	0.189	0.196
791					0.2017			0.2017		
792				0.2043	0.2033			0.2033	0.191	0.198
793					0.2049			0.2049		
794					0.2066			0.2066	0.193	0.201
795			0.194	0.2088	0.2083			0.2083		

Table 5. (Continued.)

Wavelength (nm)	Horecker (1943)	Benesch *et al* (1965)	van Assendelft (1970), Takatani and Graham (1979)	Wray *et al* (1988)	Cope (1991)	Zijlstra *et al* (1991)	Zijlstra *et al* (1994)	Matcher *et al* (1995)	Prahl (1998)	Zijlstra *et al* (2000)
796					0.2098			0.2098	0.1966	0.203
797					0.2114			0.2114		
798				0.2130	0.2131			0.2131	0.2018	0.206
799					0.2147			0.2147		
800					0.2163	0.2	0.21	0.2163	0.204	0.208
801				0.2173	0.2179			0.2179		
802					0.2195			0.2195	0.207	
803					0.2211			0.2211		
804					0.2227			0.2227	0.209	
805			0.22	0.2215	0.2243	0.21		0.2243		0.207
806					0.2259			0.2259	0.211	
807					0.2276			0.2276		
808				0.2258	0.2291			0.2291	0.214	
809					0.2307			0.2307		
810					0.2323			0.2323	0.216	
811				0.2300	0.2339			0.2339		
812					0.2354			0.2354	0.218	
813					0.2369			0.2369		
814				0.2340	0.2385			0.2385	0.22	
815			0.22		0.2401			0.2401		
816					0.2416			0.2416	0.2218	
817					0.2432			0.2432		
818				0.2380	0.2447			0.2447	0.2254	
819					0.2462			0.2462		
820					0.2478			0.2478	0.229	
821				0.2420	0.2493			0.2493		
822					0.2508			0.2508	0.2326	
823					0.2523			0.2523		
824				0.2458	0.2538			0.2538	0.2362	
825			0.238		0.2553			0.2553		
826					0.2568			0.2568	0.2391	
827					0.2583			0.2583		
828				0.2495	0.2598			0.2598	0.2413	
829					0.2612			0.2612		
830					0.2627			0.2627	0.2435	
831				0.2533	0.2641			0.2641		
832					0.2655			0.2655	0.2457	
833					0.2670			0.2670		
834				0.2570	0.2685			0.2685	0.2479	
835			0.249		0.2699			0.2699		
836					0.2713			0.2713	0.2503	
837				0.2608	0.2727			0.2727		
838					0.2741			0.2741	0.2529	
839					0.2754			0.2754		
840				0.2643	0.2768	0.25		0.2768	0.2555	0.248
841					0.2782			0.2782		
842					0.2796			0.2796	0.2581	
843					0.2808			0.2808		
844				0.2675	0.2821			0.2821	0.2607	

Table 5. (Continued.)

Wavelength (nm)	Horecker (1943)	Benesch *et al* (1965)	van Assendelft (1970), Takatani and Graham (1979)	Wray *et al* (1988)	Cope (1991)	Zijlstra *et al* (1991)	Zijlstra *et al* (1994)	Matcher *et al* (1995)	Prahl (1998)	Zijlstra *et al* (2000)
845			0.262		0.2835	0.25		0.2835		0.253
846					0.2848			0.2848	0.2625	
847				0.2710	0.2860			0.2860		
848					0.2873			0.2873	0.2635	
849					0.2887			0.2887		
850				0.2743	0.2899			0.2899	0.2645	
851					0.2911			0.2911		
852					0.2924			0.2924	0.2655	
853				0.2775	0.2936			0.2936		
854					0.2948			0.2948	0.2665	
855			0.267		0.2960			0.2960		
856					0.2971			0.2971	0.2682	
857				0.2805	0.2984			0.2984		
858					0.2995			0.2995	0.2706	
859					0.3007			0.3007		
860				0.2835	0.3018			0.3018	0.273	
861					0.3028			0.3028		
862					0.3039			0.3039	0.2754	
863				0.2865	0.3050			0.3050		
864					0.3061			0.3061	0.2778	
865			0.279		0.3071			0.3071		
866				0.2893	0.3081			0.3081	0.2796	
867					0.3092			0.3092		
868					0.3102			0.3102	0.2808	
869				0.2918	0.3112			0.3112		
870					0.3123			0.3123	0.282	
871					0.3132			0.3132		
872				0.2943	0.3141			0.3141	0.2832	
873					0.3150			0.3150		
874					0.3160			0.3160	0.2844	
875			0.285		0.3170			0.3170		
876				0.2968	0.3177			0.3177	0.2857	
877					0.3186			0.3186		
878					0.3195			0.3195	0.2871	
879				0.2990	0.3204			0.3204		
880					0.3211	0.28		0.3211	0.2885	0.284
881					0.3219			0.3219		
882				0.3010	0.3227			0.3227	0.2899	
883					0.3234			0.3234		
884					0.3243			0.3243	0.2913	
885			0.292	0.3030	0.3250			0.3250		
886					0.3257			0.3257	0.2925	
887					0.3265			0.3265		
888				0.3048	0.3271			0.3271	0.2935	
889					0.3277			0.3277		
890					0.3284			0.3284	0.2945	
891				0.3065	0.3291			0.3291		
892					0.3297			0.3297	0.2955	

Table 5. (Continued.)

Wavelength (nm)	Horecker (1943)	Benesch *et al* (1965)	van Assendelft (1970), Takatani and Graham (1979)	Wray *et al* (1988)	Cope (1991)	Zijlstra *et al* (1991)	Zijlstra *et al* (1994)	Matcher *et al* (1995)	Prahl (1998)	Zijlstra *et al* (2000)
893					0.3302			0.3302		
894					0.3309			0.3309	0.2965	
895			0.297	0.3080	0.3316			0.3316		
896					0.3321			0.3321	0.2975	
897					0.3326			0.3326		
898				0.3095	0.3331			0.3331	0.2985	
899					0.3335			0.3335		
900					0.3341			0.3341	0.2995	

$$\frac{\Delta\{\Delta[\mathrm{Hb_{total}}]\}}{\Delta[\mathrm{Hb_{total}}]} = \frac{0.80}{25.65} \times 100\% = 3.1\%. \tag{29}$$

While the relative errors caused by temperature variation from 20 to 40 °C are less than 10%, they are notable and need to be considered as possible error sources. On the other hand, such errors can be minimized by choosing proper wavelengths. Around 735, 770 and 800 nm, deoxyhaemoglobin extinction coefficients change little as temperature varies. In the range from 750 nm to 810 nm, the changes of oxyhaemoglobin extinction coefficients due to temperature variation from 20 to 40 °C are less than 0.008 $\mathrm{cm^{-1}\ mM^{-1}}$. These facts suggest that with the proper selection of wavelengths, we may be able to reduce the errors of [Hb], $[\mathrm{HbO_2}]$ and $[\mathrm{Hb_{total}}]$ induced by temperature variations.

After close inspection of the published literature, we have noted that the haemoglobin extinction coefficients, even reported from the same group at different times, can vary within a certain degree. For instance, the haemoglobin ε values from Zijlstra *et al* (1991, 1994, 2000) are not the same, as shown in table 1. Although such variation seems to be small, it introduces appreciable uncertainty in calculations of Δ[Hb], $\Delta[\mathrm{HbO_2}]$ and $\Delta[\mathrm{Hb_{total}}]$. Similarly, Wray *et al* (1988) and Cope (1991) provided different values of haemoglobin extinction coefficients. Their deoxyhaemoglobin extinction coefficients are similar to each other, while their oxyhaemoglobin extinction values show discrepancies. These facts basically show an existing challenge for biochemists to more accurately quantify haemoglobin extinction coefficients. Whether or not these ε values have already reached their limit of experimental accuracy remains to be seen. On the other hand, for biomedical physicists and engineers, it is important to be aware of uncertainties and errors in [Hb], $[\mathrm{HbO_2}]$ and $[\mathrm{Hb_{total}}]$ caused by variation of haemoglobin extinction coefficients.

Animals such as mice, rats, rabbits, dogs and pigs are often used as animal models in experiments for testing a new drug, understanding physiology, or investigating a specific disease. NIRS also has been applied to various animal experiments to monitor haemodynamics or to measure concentrations of haemoglobin derivatives. It has been a common practice to utilize human haemoglobin extinction coefficients to quantify concentrations of [Hb], $[\mathrm{HbO_2}]$ and $[\mathrm{Hb_{total}}]$ in animals, for example, rats (Kohl *et al* 2000), pigs (Klaessens *et al* 2003) and sheep (Newman *et al* 2001). Zijlstra *et al* have compared the haemoglobin extinction coefficients from dogs (Zijlstra and Buursma 1987) and rats (Zijlstra *et al* 1994) to the values from humans. The studies have shown that haemoglobin absorptivities of dogs and humans in the visible region do not differ significantly, while those of rats and humans are largely different from each other. A tabulated list of haemoglobin extinction coefficients is available

Table 6. Absolute differences in haemoglobin extinction coefficients between human and other species (unit: $mM^{-1}cm^{-1}$, four haems values).

Wavelength (nm)	Human versus rat		Human versus horse		Human versus pig		Human versus cow		Human versus sheep	
	$\Delta\varepsilon_{Hb}$	$\Delta\varepsilon_{HbO_2}$	$\Delta\varepsilon_{Hb}$	$\Delta\varepsilon_{HbO_2}$	$\Delta\varepsilon_{Hb}$	$\Delta\varepsilon_{HbO_2}$	$\Delta\varepsilon_{Hb}$	$\Delta\varepsilon_{HbO_2}$	$\Delta\varepsilon_{Hb}$	$\Delta\varepsilon_{HbO_2}$
630	0.272	0.324	0.156	0.136	0.396	0.292	0.264	0.184	0.48	0.376
660	0.048	0.092	0.088	0.076	0.104	0.044	0.108	0.032	0.268	0.256
700	0.016	0.016	0.076	0.02	0.112	0.016	0.1	0.012	0.244	0.204
750	0.064	0.012	0.008	0.012	0.036	0.004	0.012	0.016	0.12	0.16
800	0.008	0.02	0.048	0	0.06	0.016	0.044	0.008	0.12	0.124
805			0.032	0.024	0.028	0.028	0.044	0.016	0.08	0.1
840			0.02	0.036	0.06	0.028	0.032	0.028	0.064	0.092
845			0.008	0.032	0.048	0.028	0.02	0.028	0.052	0.092
880			0.028	0.036	0.052	0.032	0.028	0.036	0.052	0.084

(Zijlstra *et al* 2000) for cows, pigs, horses and sheep from 450 nm to 800 nm (in every 2 nm) and a few points between 800 nm to 1000 nm. In the same reference, the haemoglobin ε values for dogs were given in the range of 450 nm to 610–690 nm, while the rat haemoglobin ε values were tabulated from 450 nm to 800 nm. For comparison, we have summarized the differences in ε values between humans and other species in table 6. This table is obtained after multiplying the original ε values given by Zijlstra *et al* (2000) by a factor of 4. It is seen that the differences become smaller when wavelengths are longer than 700 nm. Thus, it is more accurate and thus preferable to utilize the haemoglobin extinction coefficients of corresponding animals for animal experiments. Especially, we suggest avoiding the use of human haemoglobin ε values for sheep measurements or for sheep blood since the relative differences of ε values between human and sheep are up to 7% to 50%, possibly leading to hidden but significant errors in calculations of haemoglobin derivative concentrations.

The effect of pH changes on methaemoglobin extinction coefficients (ε_{Hi}) has been reported by Benesch *et al* (1973) and Zijlstra *et al* (1994). Benesch *et al* (1973) reported changes of ε_{Hi} values at 540, 560, 570, 576 and 630 nm when the pH values changed from 6.2 to 8.8. Specifically, as pH increased from 6.2 to 8.8, ε_{Hi} at 630 nm increased more than 50% of those at pH = 6.2, while ε_{Hi} values at other wavelengths (540, 560, 570, 576 nm) were decreased more than 50% from its value at pH = 6.2. Zijlstra *et al* (1994) also reported the effect of pH changes on ε_{Hi} of humans and rats in the wavelength range of 450 nm to 700 nm. They found that the pH-dependence of rat ε_{Hi} was limited within pH = 6.42 and 6.9, but that human ε_{Hi} were greatly affected by pH changes from 520 to 620 nm within pH = 6.01 and 7.34. For the effect of pH on Hb and HbO_2 extinction coefficients in the NIR region, Helledie and Rolfe (1990) have reported that there is little pH effect on ε_{Hb} and ε_{HbO_2}.

In conclusion, we showed in this study that there could be a significant error in the determination of haemoglobin derivative concentrations using NIRS when the values of haemoglobin extinction coefficients have variations or uncertainties. The variations in ε values can result from the wavelength shift during the measurements, temperature deviation, and different literature sources given for the haemoglobin extinction coefficients. The mathematical calculations in combination with the blood phantom experiments demonstrated that even small discrepancies in haemoglobin extinction coefficients between different sources can cause 5–25% relative errors in quantification of haemoglobin concentrations. Our study has found that among changes in $\Delta[Hb]$, $\Delta[HbO_2]$ and $\Delta[Hb_{total}]$, the error in $\Delta[Hb_{total}]$ caused by discrepancies of haemoglobin extinction coefficients is not always larger than

errors of Δ[Hb] or Δ[HbO_2]. Although our derivations have been developed to obtain error analysis for Δ[Hb], Δ[HbO_2] and Δ[Hb_{total}], they are also valid for estimating errors in absolute concentrations of [Hb], [HbO_2] and [Hb_{total}]. We also discussed the variations in ε values due to temperature changes and possible errors induced by using human ε values for animals. We wish to suggest that readers process the raw animal data with the corresponding animal haemoglobin extinction coefficients in order to obtain accurate values of [Hb], [HbO_2] and [Hb_{total}] taken from animals. Otherwise, an alternate method is to use the closest extinction coefficients from the other species available.

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