Asian Journal of Chemistry Vol. 13, No. 4 (2001), 1500–1512

Quantitative Analysis to Calculate the Millimolar Absorptivity and the Linear Absorption Coefficient of Human Haemoglobin Derivatives

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The paper presented an experimental modification of the millimolar absorptivity of the fundamental four haemoglobin derivatives for human blood. All investigators powerfully recommended the huge warranty owing to the crucial transforming of the ferrohaemoglobin (oxyhaemoglobin) to ferrichaemoglobin (deoxyhaemoglobin), particularly after the over world-wide using of the spectrophotometer technique in diagnosing many fine and sensitive cases, in addition the using of radiotherapy and chemotherapy in many urgent and long-term treatments, as leukemia and other cancer remedy cases, in which the ferrohaemoglobin spectrum is one of the leading investigations in report on the development of the patients' cases.

The consequence of this paper can be estimated from the directly computerized approach of mutually millimolar absorpitivity and linear absorption coefficient for the majority imperative haemoglobin derivatives, implicitly, oxyhaemoglobin, and its necessary playful in the human blood to tolerate or start immediately providing the case with the required chemicals.

The attentiveness millimolar absorptivity was established by estimating the optical density of each species, at the most convenient investigated samples conditions, i.e., concentration, pH, temperature and light path, get-together of wavelength along the visible spectrum using a computerized technique which gives a direct extent of the optical density as a function of the program wavelength. The linear affiliation flanked by the optical density and the millimolar absorptivity at each wavelength to each derivative was utilized for manipulating the attentiveness of the different haemolglobin derivatives in each geared up sample. A modified urbanized computer program watchful to resolve the linear equations hold unknowns, and to find out the best-fit millimolar absorptivity of each class. The linear absorption coefficient at each wavelength was intended as a functon of together millimolar absorptivity as well as the haemoglobin species concentration. One of the significant statements of the obtainable study is, the millimolar absoptivity was almost constant, despite the fact that the linear absorption coefficient is a function of the concentration.

INTRODUCTION

Reachable optical millimolar absorptivity data reported in literature, even though very limited, no exhibit significant disagreement in some parts of the spectra. This may be due to a number of reasons including differences in the method employed in preparing the haemoglobin solutions, the blood material itself and the method used in analyzing the samples by Zijlstra[1], Burcard[2], Van Asendleft[3,4] and Phiri[5]. Therefore discretion must be exercised in comparing the

reported data with the data reported in this work, signifying very clearly haemoglobin concentration, pH, and temperature, of the investigated samples, because of their observed effects in the quantitative analysis course.

Quantitative analysis and characterization of human blood haemoglobin and its derivatives, oxyhaemoglobin, carbomonohaemoglobin, deoxyhaemoglobin and methaemoglobin, were conducted using computerized integrating sphere spectrophotometer technique[6]. The optical densities were measured in the visible wavelength range (400–700 nm) to determine the millimolar absorptivity and linear absorption coefficient values of each haemoglobin species, in order to meet the goal of this paper in the comparing method among the spectra of the species represented by wavelength against linear absorption coefficient and wavelength against millimolar absorptivity.

A computerized technique is used to solve the two hundred fifty one equations to fit the corresponding number of millimolar absorptivities, for each class individually. The process was repeated four times at the same analyzing conditions; the values appeared in this paper represent the average of the four frequent samples.

Alternatively, authors reported that light scattering could be occurred because of the molecular interactions among each other[7,8], while others reported that a minor scattering, not more than 1%, dominate during the spectrophotometer measurement[2]. Integrating sphere spectrophotometer was used to completely eliminate the scattering process effects, so very nice curves of the four haemoglobin derivatives, representing the relation between the absorption coefficient and wavelength, were plotted, using the known relation,

$$\mu_a = \log [\varepsilon(\lambda)C] \tag{1}$$

where μ_a is the linear absorption coefficient, measured in cm^{-1}, of the molecules, $\varepsilon(\lambda)$ is the millimolar absorptivity measured in L/mmol.cm, and C is the concentration of the sample, measured in mmol/L.

Millimolar absorptivity, for each class, was premeditated using a straightforward and unwavering computer program, constructed on the singular value decomposition to work out the two hundred fifty one equations in the visible light spectrum, to each haemoglobin category, *i.e.*, HbO_2, HiHb, CoHb and Hb.

$$\begin{aligned} OD_{450} &= \varepsilon_{450} C_{HbO_2} \\ &\vdots \quad\quad \vdots \\ OD_{700} &= \varepsilon_{700} C_{HbO_2} \end{aligned} \tag{2}$$

The same procedure was frequent for the other three haemoglobin categories. A chief significance be supposed to identified here, that is, the concentration of all other species in each analyzed case is move toward zero, to guarantee that the haemoglobin species was uncontaminated, unchanged, which is extremely having an essential concern in assessment of the millimolar absorptivity.

Four Haemoglobin Species Groundwork and Inspection

The total haemoglobin attentiveness (C_t) was intended after diluting the

concentrated substance with the reagent solution. The reagent was prepared by dissolving 200 mg of $K_3Fe(CN)_6$, 50 mg of KCN, and 140 mg of KH_2PO_4 in distilled deionized water and made up to one litre, the pH was adjusted to 7.4 and the solution kept in brown borosilicate container. The molar attentiveness was determined using the equation:

$$C_t = [OD \cdot F/\varepsilon(\lambda)L] \quad (3)$$

where F is the dilution factor (220 : 1), OD is the optical density at 540 nm; ε is the value of the quarter millimolar extinction coefficient of haemoglobin cyanide at the wavelength 540 nm which equals 11.5 L/mmol cm and L is the light path; equal to 1.00 cm. The dilution factor was adjusted with the optical density, which is about 0.435 acceptable to keep the inaccuracy in C_t at a minimum. The unyielding RBC was diluted to different concentrations in turn to identify the optimal concentration range for spectrophotometric assay[6].

The prepared oxyhaemoglobin was examined throughout the developed spectroscopic computerized technique, the optical densities were feeding the ready subroutine to evaluate the two hundred fifty one unknown millimolar absorptivity, starting from 450 nm to 700 nm, covering the effective range in the visible light spectrum. Fig. 1 shows both of the millmolar absorptivity and the linear absorption coefficient as a function of the wavelength, at selected convenient configuration values. For more details, the calculated values are given in, Table-1. It is clear that most effective values have been chosen to obtain the reliable configuration of the spectrum.

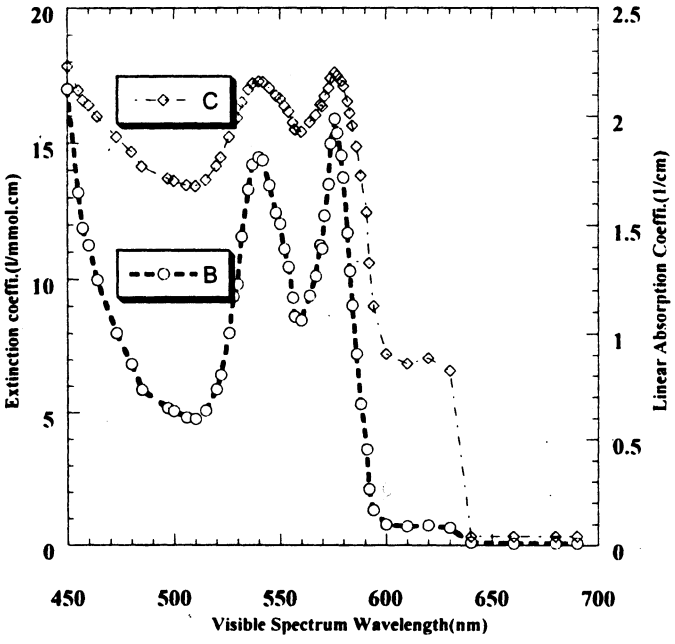

Fig. 1. Oxyhaemoglobin millimolar absorpitivity (B) and absorption coefficient (C) as a function of wavelength.

TABLE-1
THE VISIBLE SPECTRUM WAVELENGTHS AND THEIR CORRESPONDING VALUES OF MILLIMOLAR ABSORPTIVITY AND LINEAR ABSORPTION COEFFICIENT, FOR HbO_2

No.	Wavelength (nm)	$\varepsilon(\lambda)$ Millimolar absorptivity (L/mmol.cm)	μ_a Absorption coefficient (L/cm)
1.	450	17.000	2.2304
2.	455	13.200	2.1205
3.	457	11.900	2.0755
4.	460	11.260	2.0515
5.	464	9.9800	1.9991
6.	473	8.0100	1.9036
7.	480	6.8400	1.8350
8.	485	5.8600	1.7678
9.	497	5.1600	1.7126
10.	500	5.0500	1.7032
11.	506	4.8100	1.6821
12.	510	4.7600	1.6776
13.	515	5.0800	1.7058
14.	520	5.8800	1.7693
15.	522	6.4200	1.8088
16.	526	8.0000	1.9030
17.	528	9.3500	1.9708
18.	530	9.8000	1.9912
19.	532	11.590	2.0640
20.	535	13.300	2.1238
21.	537	14.210	2.1525
22.	540	14.470	2.1604
23.	542	14.370	2.1574
24.	545	13.450	2.1287
25.	548	12.460	2.0955
26.	550	12.060	2.0813
27.	552	11.120	2.0461
28.	554	10.460	2.0195
29.	556	9.3200	1.9694
30.	557	8.6200	1.9355
31.	560	8.4700	1.9278
32.	564	9.3800	1.9722

No.	Wavelength (nm)	ε(λ) Millimolar absorptivity (L/mmol.cm)	μ_a Absorption coefficient (L/cm)
33.	567	10.120	2.0051
34.	569	11.270	2.0519
35.	570	11.140	2.0468
36.	571	12.360	2.0920
37.	573	13.500	2.1303
38.	574	14.980	2.1755
39.	576	15.900	2.2013
40.	577	15.370	2.1866
41.	579	14.550	2.1628
42.	580	13.730	2.1376
43.	582	11.720	2.0689
44.	583	10.320	2.0136
45.	584	9.0300	1.9556
46.	586	7.2300	1.8591
47.	588	5.3210	1.7259
48.	591	3.6200	1.5587
49.	592	2.1300	1.3283
50.	594	1.3400	1.1271
51.	600	0.8000	0.9030
52.	610	0.7210	0.8579
53.	620	0.7650	0.8836
54.	630	0.6700	0.8260
55.	640	0.1100	0.0413
56.	660	0.0800	0.0413
57.	680	0.0800	0.0413
58.	690	0.0700	0.0413

Methaemoglobin was prepared by adding 30 mg of potassium ferricyanide to 10 mL of concentrated fresh oxyhaemoglobin. The ferricyanide acts to oxidize the oxyhaemoglobin to methaemoglobin. The excess ferricyanide was then removed using a Sephadex G-25 sieving column eluted with washing buffer. The product was collected by an autofractioner in test tube analyzer spectrophotometrically to determine the methaemoglobin fraction. The second step was to concentrate the dilute methaemoglobin using the dialysis method. Fig. 2 shows both of the millimolar absorptivity and the linear absorption coefficient as a function of the wavelength. For more details, the calculated values are given in Table-2.

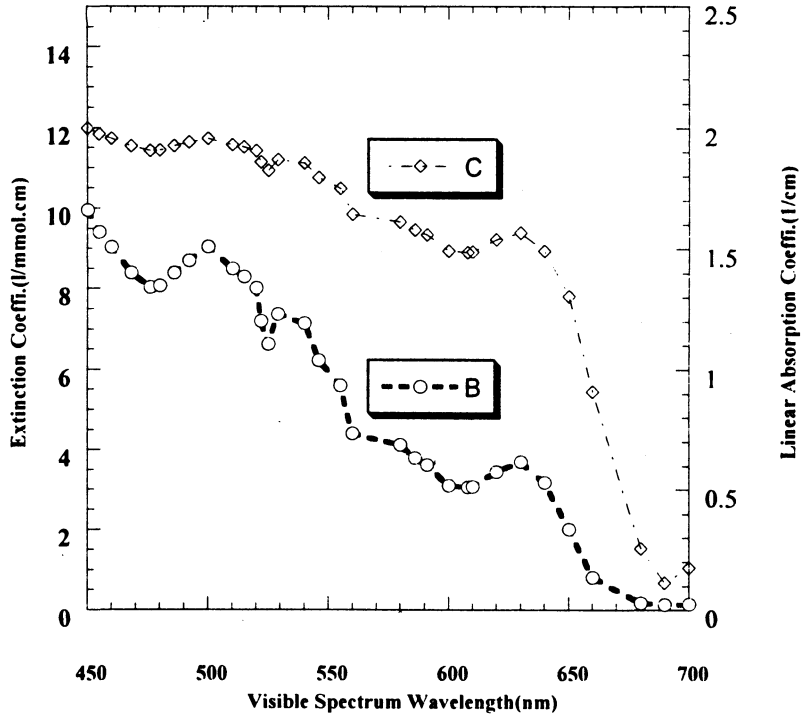

Fig. 2. Methaemoglobin millmolar absorpitivity (B) and absorption coefficient as a function of wavelength

TABLE-2
THE VISIBLE SPECTRUM WAVELENGTHS AND THEIR CORRESPONDING VALUES OF MILLIMOLAR ABSORPTIVITY AND THE LINEAR ABSORPTION COEFFICIENT OF HUMAN HAEMOGLOBIN DERIVATIVES

No.	Wavelength (nm)	$\varepsilon(\lambda)$ Millimolar absorptivity (L/mmol.cm)	μ_a Absorption coefficient (L/cm)
1.	450	9.9500	1.9978
2.	455	9.4000	1.9731
3.	460	9.0300	1.9556
4.	468	8.4000	1.9242
5.	476	8.0400	1.9052
6.	480	8.0700	1.9069
7.	486	8.4000	1.9242
8.	492	8.7000	1.9395

No.	Wavelength (nm)	ε(λ) Millimolar absorptivity (L/mmol.cm)	μ_a Absorption coefficient (L/cm)
9.	500	9.0400	1.9561
10.	510	8.5000	1.9294
11.	515	8.3000	1.9197
12.	520	8.0200	1.9041
13.	522	7.2000	1.8573
14.	525	6.6200	1.8208
15.	529	7.3700	1.8674
16.	540	7.1400	1.8536
17.	546	6.2300	1.7940
18.	555	5.6000	1.7480
19.	560	4.4000	1.6434
20.	580	4.1200	1.6127
21.	586	3.7900	1.5786
22.	591	3.6200	1.5587
23.	600	3.1000	1.4913
24.	608	3.0600	1.4857
25.	610	3.0800	1.4885
26.	620	3.4500	1.5378
27.	630	3.7000	1.5682
28.	640	3.1800	1.4913
29.	650	2.0100	1.3031
30.	660	0.8100	0.9084
31.	680	0.1800	0.2552
32.	690	0.1300	0.1139
33.	700	0.1500	0.1760

The ferrihaemoglobin was prepared by mixing 4 mg of sodium dithionite crystals with 10 mL fresh oxyhaemoglobin. Reducing the oxyhaemoglobin to ferrihaemoglobin oxidized sodium dithionite. Fig. 3 shows both of the millimolar absorptivity and the linear absorption coefficient as a function of the wavelength. For more details, the calculated values are given in Table-3.

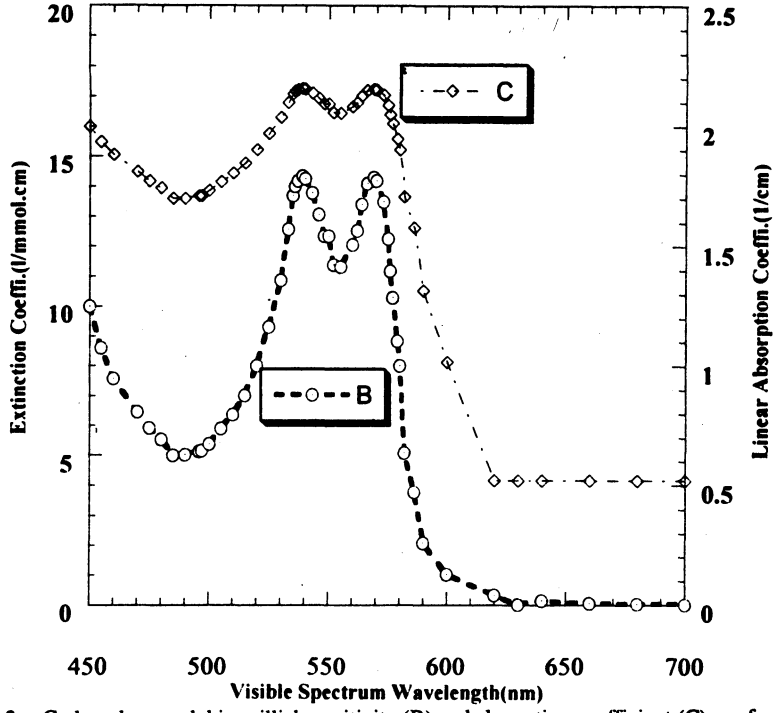

Fig. 3. Carboxyhaemoglobin milliabsorpitivity (B) and absorption coefficient (C) as function of wavelength.

TABLE-3
THE VISIBLE SPECTRUM WAVELENGTHS AND THEIR CORRESPONDING VALUES OF MILLIMOLAR ABSORPTIVITY AND LINEAR ABSORPTION COEFFICIENT, FOR CARBOXYHAEMOGLOBIN

No.	Wavelength (nm)	$\varepsilon(\lambda)$ Millimolar absorptivity (L/mmol.cm)	μ_a Absorption coefficient (L/cm)
1.	450	10.0000	2.0000
2.	455	8.6100	1.9350
3.	460	7.5900	1.8800
4.	470	6.4600	1.8100
5.	475	5.9300	1.7730
6.	480	5.5400	1.7435
7.	485	5.0000	1.6989
8.	490	5.0200	1.7007
9.	496	5.1400	1.7109
10.	497	5.1600	1.7120
11.	500	5.4000	1.732
12.	505	5.9000	
13.	510	6.3800	
14.	515	7.0200	

No.	Wavelength (nm)	ε(λ) Millimolar absorptivity (L/mmol.cm)	μ_a Absorption coefficient (L/cm)
15.	520	8.0200	1.9041
16.	525	9.3300	1.9698
17.	530	10.9000	2.0374
18.	533	12.6000	2.1003
19.	535	13.7000	2.1367
20.	536	14.0200	2.1467
21.	537	14.2000	2.1523
22.	539	14.3600	2.1571
23.	540	14.2700	2.1544
24.	543	13.8000	2.1398
25.	546	13.0800	2.1166
26.	548	12.3700	2.0923
27.	550	12.3700	2.0923
28.	552	11.4100	2.0572
29.	555	11.3300	2.0542
30.	560	12.0800	2.0821
31.	562	12.5400	2.0982
32.	564	13.4100	2.1274
33.	566	14.1000	2.1492
34.	569	14.3100	2.1556
35.	570	14.2000	2.1522
36.	573	13.5000	2.1303
37.	575	12.2800	2.0891
38.	576	11.2000	2.0492
39.	577	10.3200	2.0136
40.	579	8.8600	1.9474
41.	580	8.0200	1.9042
42.	582	5.1000	1.7075
43.	586	3.7900	1.5786
44.	590	2.0700	1.3159
45.	600	1.0400	1.0170
46.	620	0.3300	0.5185
47.	630	0.0200	0.5185
	640	0.1500	0.5185
	660	0.0700	0.5185
	680	0.0400	0.5185
	700	0.0200	0.5185

axide haemoglobin was prepared by exposing 4 mL of freshly aemoglobin to carbon monoxide, a cuvette equipped with

a magnetic stirrer and sealed with airtight septum; carbon monoxide was introduced to the cuvette from cylinder connected with the cuvette, while the oxyhaemoglobin was stirred. Fig. 4 shows both of the millimolar absorptivity and the linear absorption coefficient as a function of the wavelength. For more details, the calculated values are given in Table-4.

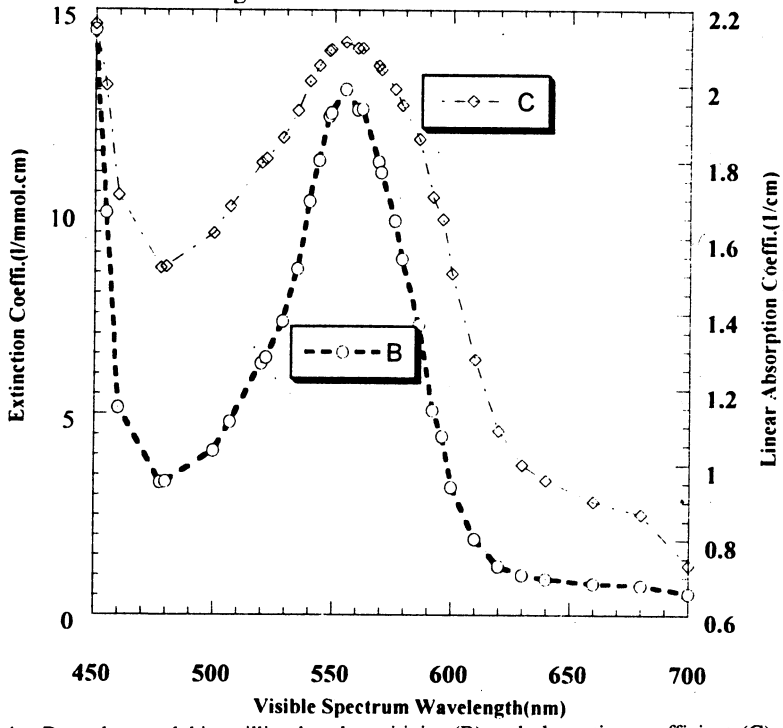

Fig. 4. Deoxyhaemoglobin millimolar absorpitivity (B) and absorption coefficient (C) as a function of wavelength.

TABLE-4
THE VISIBLE SPECTRUM WAVELENGTHS AND THEIR CORRESPONDING VALUES OF MILLIMOLAR ABSORPTIVITY AND LINEAR ABSORPTION COEFFICIENT FOR Hb

No.	Wavelength (nm)	$\varepsilon(\lambda)$ Millimolar absorpitivity (L/mmol.cm)	μ_a Absorption coefficient (L/cm)
1.	450	14.500	2.1613
2.	455	10.000	2.0000
3.	460	5.150	1.7118
4.	478	3.310	1.5198
5.	480	3.340	1.5237
6.	500	4.090	1.6117
7.	507	4.810	1.6821
8.	520	6.270	1.7972
9.	522	6.420	1.8075

No.	Wavelength (nm)	ε(λ) Millimolar absorpitivity (L/mmol.cm)	μ_a Absorption coefficient (L/cm)
10.	529	7.310	1.8639
11.	535	8.600	1.9344
12.	540	10.280	2.0119
13.	544	11.300	2.0530
14.	548	12.370	2.0924
15.	549	12.460	2.0955
16.	555	13.040	2.1150
17.	560	12.540	2.0982
18.	562	12.560	2.0989
19.	569	11.270	2.0519
20.	570	11.000	2.0414
21.	576	9.800	1.9912
22.	579	8.860	1.9474
23.	586	7.230	1.8590
24.	592	5.100	1.7075
25.	596	4.460	1.6493
26.	600	3.200	1.5051
27.	610	1.900	1.2787
28.	620	1.230	1.0899
29.	630	1.000	1.0000
30.	640	0.910	0.9590
31.	660	0.800	0.9030
32.	680	0.740	0.8692
33.	700	0.540	0.7323

All of the four derivatives were examined very precisely using the described spectroscopic computerized technique[6].

RESULTS AND DISCUSSION

Many published articles about the measurements of human blood haemoglobin and its derivatives indicated that the millimolar absorptivity for the different classes is not affected evidently by the concentration of the haemoglobin[1]; others stated the moderate total haemoglobin concentrations 0.01–0.1 mmol L^{-1}, *i.e.*, in the concentration range at which their own values agree with those of the other authors[2, 4, 9, 10]. The results in the present work are fully in agreement with these results, in spite of that the chosen concentration is 10 mmol L^{-1}. This observation can be explained by the following assumption: in the visible wavelength region only the intensity of the absorption band changes with haemoglobin concentration. Keeping in mind the theoretical consideration given by the equation described the operator for the molecule dipole moment, which is explaining the influence by the concentration mechanism[2]; It is very imperative to assure that

the energy difference between the ground state and the excited state does not change, this is the convenience and matrimonial reality helping in clearing up the end product of the studied sample concentration on the millimolar absorptivity.

Other publications point out that the concentration performs its full-size influence on the intended values of the millimolar absorptivity; consequently they have found the values at the high and low concentrations, finally, taking into account the average values. In conclusion, they end up with approximately the same results.[11]

On the other hand, scattered light outcome on the quantity, which has been discussed in many published papers[2], is completely excluded in the present work, throughout using the integrating sphere spectroscopic technique[6].

In the present work the concentration (10 mmol L^{-1}) has been chosen for the very obviously spectral performance of the millimolar absorpitivity of oxyhaemoglobin, over and above the other haemoglobin species. Fig. 1 displays the visible spectrum wavelength as a function of the millimolar absorptivity, representing the different locations of upper and lower limits.

In reality, the effective factor in the whole studied concentration is the linear absorption coefficient equation (1) expressed the intended state of affairs. Fig. 1 also put on view the dependence of this coefficient on the haemoglobin concentration. For the purpose of comparison, the chosen value was 10 mmol L^{-1}. Figure 1 is an obvious computerized measurement of together millimolar absorptivity and linear absorption coefficient as a function of visible wavelength. On this occasion the comparison clearly shows the overall similarity in shape, in addition to the near adaptation in the locations of both upper and lower limits of wavelength.

Many other publications discussed the observed impact of the haemoglobin pH on the calculated values of millimolar absorptivity[11], but most of them[6, 12] agreed that the value 7.4 is the most appropriate one; because of this, it is the used value in this work.

The considered values of temperature in most of the previous publications were alternating between 37°C and 24°C. In the present work the temperature 24°C is recommended in any other further future investigations.

Table-1 shows numerically the values for together millimolar absorptivity and linear absorption coefficients of the oxyhaemoglobin at the matching wavelength values.

Figs, 2–4 respectively put on view the dependence of millimolar absorptivity and linear absorption coefficients of methaemoglobin, carboxyhaemoglobin and deoxyhaemoglobin, at the same value of concentration. These figures are obvious quantitative analysis measurements of together millimolar absorptitvity and linear absorption coefficient as a function of visible wavelength. In this justification, the comparison clearly shows the overall similarity in the shape, in addition to the near acclimatization in the positions of both higher and lesser limits of wavelength.

In conclusion, these reliable figures introduce new and feasible spectra of the four most significant haemoglobin categories, at reliable research circumstances,

i.e., temperature, concentration and pH values, using a very sophisticated quantitative analysis technique.

Tables 1 to 4 show numerically the calculated values for together millimolar absorptivity and linear absorption coefficients of methaemoglobin, carboxyhaemoglobin and deoxyhaemoglobin at the matching wavelength values.

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(*Received:* 17 May 2001; *Accepted:* 14 August 2001) AJC-2404