

# **Measurement of Temperature and Concentration Dependences of Refractive Index of Hen-egg-white Lysozyme Solution**

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## **Measurement of Temperature and Concentration Dependences of Refractive Index of Hen-egg-white Lysozyme Solution**

This paper reports the measurement of temperature and concentration dependences of the refractive index of hen-egg-white lysozyme (HEWL) solution, and the temperature dependence of refractive index of the quartz container, which are indispensable for the efforts to measure HEWL concentration of the solution during the processes like crystal growth and dissolution using interferometer. As all of these dependences might be different under different observation wavelengths, temperatures and solution compositions, it is necessary to measure them for specific conditions. In this paper, the desired dependences were measured without knowing the actual value of the refractive index. The measurement method was introduced and the measurement experiments were undertaken at wavelength 780nm. The dependences obtained will be also useful for those studies employing the same wavelength and solution conditions.

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### **1. Introduction**

The variation of solute concentration during the growth process of protein crystal can provide important information for understanding the phenomena like mass transfer in the process; therefore it is very useful to explore methods of monitoring concentration change of protein solution during the crystal growth process. Many attempts have been made to determine the concentration of protein in a solution. For the purpose of the protein

concentration determination in a solution, we can find numerous assay techniques in the literatures [1-11]. However, for the purpose of protein crystal growth study, some in-situ monitoring techniques are more competitive. Raman spectroscopy was recently used for real-time monitoring of the hen-egg-white lysozyme (HEWL) concentration during crystallization [12]; more recently, NIR (near infrared) method was utilized to measure the protein concentration [13]. For more detailed concentration mapping, interferometry was proposed to be a more convenient tool by Shlichta [14] in 1980s. It has been proved to be an effective method to in-situ measure the concentration distribution profile of protein in the solution [15-22].

For the concentration measurement method using interferometer, some physical parameters are indispensable because the measurement is based on the relationship between the refractive index of the solution and the concentration of the solute in the solution. For the case when the solution is kept under a constant temperature, the dependence of the refractive index of the solution on the concentration ( $[\partial n_s / \partial C]_T$ ) should be known. If in a case when the temperature changes during the process, other two parameters are necessary, *i.e.*, the dependence of the refractive index of protein solution on temperature ( $[\partial n_s / \partial T]_C$ ), and the dependence of the refractive index of the container on temperature ( $[\partial n_q / \partial T]$ ).

In the literature, Fredericks *et al.* gave a comprehensive report about the physical properties of HEWL solution [23], the dependences of refractive index of the solution (at the sodium D-line, 589 nm) on temperature and on concentration were also included. Other researchers [16-20, 24] also reported their own results of the concentration dependences of refractive index of HEWL solution under different conditions. However, if we hope to study the concentration change during the process while the temperature varying with time, the data provided in the literatures (mostly suitable for isothermal process) are not enough for the purpose. Furthermore, the actual experimental conditions are different in that we used different wavelength laser source, and the solution doesn't contain buffer. Therefore it is necessary to make measurements of the parameters mentioned above in our own conditions.

As to the measurement method, some efforts have been made in the literature. For example, recently, Kekicheff *et al* [25] measured the dependence of the refractive index on the concentration of cytochrome *c* using a combination of an interferometer for determination of refractive index of the solution and a near infrared microspectroscopy for determination of water during slow evaporation of the solvent. Others used Abbe refractometer [20-22, 26], Reileigh interferometer [24] to determine the parameters. In the current work, we used a Mach-Zehnder interferometer to measure the parameters without knowing the actual value of the refractive index. The measurement techniques will be introduced in the following section. Such measurement technique will be also useful for the determination of similar physical parameters in other solution systems.

## 2. Measurement method

### 2.1. Principle

For our experimental conditions, as the thickness of the solution in the optical pathway is very thin (1mm), we assumed that the solution different in the direction of the optical pathway is negligible. In such case we can treat the problem two dimensionally so that every point in the observed region can be expressed by an orthogonal coordinate  $(x,y)$ . (See Fig 1, the schematic illustration of the studied cell).

If we study the solution only, and suppose that the initial refractive index of the solution is  $n_0$ , after a short period of time  $Dt$  the refractive index changes to  $n(x,y)$  at the point  $(x,y)$  for some reasons (temperature change and/or concentration change). If the phase change at point  $(x,y)$  is  $Df(x,y)$ , then we have:

$$[n(x, y) - n_0]d = \frac{I}{2p} \Delta f(x, y), \quad (1)$$

where  $I$  is the wavelength of the light beam,  $d$ : the thickness of the solution in the direction of the optical pathway.

At a certain wavelength, the refractive index is a function of temperature and concentration [27]:

$$\Delta n = n(x, y) - n_0 \approx \left( \frac{\partial n}{\partial C} \right)_T \Delta C(x, y) + \left( \frac{\partial n}{\partial T} \right)_C \Delta T(x, y), \quad (2)$$

where  $DC(x,y)$  and  $DT(x,y)$  are concentration and temperature changes at point  $(x,y)$ , respectively.

Thus

$$\left\{ \left[ \frac{\partial n}{\partial C} \right]_T [C(x, y) - C_0] + \left[ \frac{\partial n}{\partial T} \right]_C [T(x, y) - T_0] \right\} d = \frac{I}{2p} \Delta f(x, y), \quad (3)$$

where  $C_0$ : initial concentration,  $T_0$ : initial temperature,  $T(x,y)$ : temperature at  $(x,y)$ ;  $C(x,y)$ : concentration at  $(x,y)$ .

For the studied point, if we let  $DT(x,y)=0$ , through measuring the concentration change  $DC(x,y)$  by spectrophotometry and the phase change  $Df(x,y)$  by interferometry, we can obtain  $[\partial n / \partial T]_C$  according to Eq. (3); if we try to avoid crystallization so as to keep concentration constant in a reasonable short time, *i.e.*,  $DC=0$ , while changing the temperature and recording the phase change  $Df(x,y)$  at the same time, then we can obtain  $[\partial n / \partial C]_T$  from Eq. (3). This is the principle for the measurements.

### 2.2. Measurement of the temperature dependence of refractive index of quartz $[dn_q/dT]$

For correct measurement we must first obtain the optical constant of the quartz container because the laser beam will pass through both the solution and the container. The term “quartz” can be misleading because many forms of silica are referred to as “quartz”, such as fused quartz, synthetic quartz, crystal quartz (rock quartz), quartz glass, *etc.* Some physical properties of these types of “quartz” are quite different from each other due to the structure difference despite that they all composed or mainly composed of silicon dioxide. As to the temperature dependence of refractive index of quartz, according to the literatures, the parameters provided vary from each other depending on the wavelength and actual types of quartz. For example, DeNicola *et al.* [28] reported two negative parameters of the ordinary and extraordinary refractive indices variation versus temperature for crystal quartz  $dn_o/dT=-7.2\times 10^{-6}/K$  and  $dn_e/dT=-8.8\times 10^{-6}/K$  at wavelength 632.8nm. Lukin et al [26] reported a positive value  $dn/dT=6.20\times 10^{-6}/K$  at wavelength 632.8nm for fused quartz. The differences are so large that it is hard to choose a datum from the literatures for our purpose. Furthermore, there are no reference data available in the literature at wavelength 780 nm which we used for the concentration measurement. Therefore we have to try to measure the parameter by ourselves.

As refractive index of the quartz is a function of temperature, so if the laser beam passes through the quartz only (at the lower part of the cell, for example) and if we change the temperature of the quartz, the fringe will shift  $Df_q(x,y)$  according to the following equation:

$$\left[ \frac{\partial n_q}{\partial T} \right] \cdot [T(x, y) - T_0] \cdot l = \frac{l}{2p} \Delta f_q(x, y). \quad (4)$$

If the environment is stable, we may be able to measure  $[\partial n_q/\partial T]$  directly according to the above equation. However, for more reliable results we must consider the effect of the environmental reasons. Environment induced fringe shift might be always varying irregularly due to the environmental instabilities such as temperature fluctuation in the optical system, air disturbances, or other reasons, which make it difficult to determine the actual value of  $[\partial n_q/\partial T]$  directly.

Fortunately we can study the small region around the cell bottom, where the laser passes through two regions:  $\rightarrow$ quartz $\rightarrow$ solution $\rightarrow$ quartz (at the upper part of the observation region), and  $\rightarrow$ quartz only (at the lower part of the observation region). The whole observation region is very small (less than  $4.27\times 5.39\text{mm}^2$ ) so that we can take the environment induced phase shift as the same at both parts near the bottom  $Df_e(x,y)=Df_e$ . If we put pure water (because we know very well about the optical data of water) into the cell, and study the both regions by varying the temperature of the cell, through comparing of the fringe shifts at both regions we can eliminate the errors caused by the environmental reasons.

$$\left[ \frac{\partial n_q}{\partial T} \right] = \frac{1}{2p\Delta Td} (\Delta \mathbf{f}_{Lower} - \Delta \mathbf{f}_{Upper} + \Delta \mathbf{f}_{H_2O}) \quad (5)$$

where  $\Delta \mathbf{f}_{Lower}$ : phase shift at the lower part,  $\Delta \mathbf{f}_{Upper}$ : phase shift at the upper part, and  $\Delta \mathbf{f}_{H_2O}$ : phase shift caused by water, which can be calculated according to the data provided in the literature [29]. At a certain wavelength, and in a small temperature range (0~60°C), we take  $[\partial n_q / \partial T]$  as a constant  $[dn_q/dT]$  to express the temperature dependence of the refractive index of the quartz cell.

### 2.3. Measurement of $[\partial n_s / \partial C]_T$ and $[\partial n_s / \partial T]_C$

In an actual system, in addition to the refractive index change of the solution, we must also consider the effect of quartz cell and the environment effect on the phase change during the measurement. When there is a temperature change in the quartz cell, the refractive index of the cell will be changed, thus the phase shift in the observed region will be influenced. Environmental reasons will also contribute to the phase shift in the observed region as described in the above section. Thus the total phase shift of the observed point  $(x,y)$  in an actual measurement system can be written as follow:

$$\Delta \mathbf{f}(x, y) = \Delta \mathbf{f}_q(x, y) + \Delta \mathbf{f}_s(x, y) + \Delta \mathbf{f}_e(x, y), \quad (6)$$

where  $\Delta \mathbf{f}_q(x,y)$ : the phase shift caused by the refractive index change of quartz,  $\Delta \mathbf{f}_s(x,y)$ : the phase shift caused by the solution, and  $\Delta \mathbf{f}_e(x,y)$ : the phase shift caused by the environmental reasons. Using the same method to eliminate the environment induced errors described in section 2.2, we can obtain  $[\partial n_s / \partial C]_T$  and  $[\partial n_s / \partial T]_C$  through the following equations:

$$\left[ \frac{\partial n}{\partial T} \right]_C = \left[ \frac{\partial n}{\partial T} \right]_q + \frac{1}{2p\Delta Td} (\Delta \mathbf{f}_{Upper} - \Delta \mathbf{f}_{Lower}) \quad (7)$$

$$\left[ \frac{\partial n}{\partial C} \right]_T = \frac{1}{2p\Delta Cd} (\Delta \mathbf{f}_{Upper} - \Delta \mathbf{f}_{Lower}) \quad (8)$$

## 3. Experimental

A Mach-Zehnder interferometer was utilized to observe the refractive index change of the solution. Fig 2 (a) and (b) show schematically the experimental setups for the measurement. In Fig 2 (a), BS1~BS2 are beam splitters, M1~M2 are mirrors. The sample cell was fixed to the interferometer using a sample holder, which is a combination of a pair of Peltier devices and a water-cooling system (Fig. 2 (b)). Temperatures of the upper and lower part of the sample cell could be controlled by the Peltier devices with a high temperature control accuracy

(up to  $\pm 0.1^\circ\text{C}$ ) separately in the range of  $0\sim 60^\circ\text{C}$ . In the current study we used isothermal temperature control only for the measurement convenience. The interferograms were captured by a CCD camera and recorded by a videocassette recorder (VCR) for final analysis. The light source of the interferometer was a laser diode (LD in Fig.2 (a)) at wavelength 780nm.

The solutions were prepared at different initial HEWL (Six times recrystallized HEWL from Seikagaka Kogyo) concentrations ranging from 0mg/ml to 70mg/ml for the experimental runs. Other conditions of the solutions are: pH=4.60, NaCl: 40mg/ml.

## 4. Results and discussions

### 4.1. $[dn_q/dT]$

Table 1 listed the data reported in the literatures and our measurement result. From the table we can see that  $[dn_q/dT]$  of the crystalline quartz are negative, while that of the amorphous quartz is positive. To make comparisons we also listed some reports for fused silica and silica glass which are very similar to fused quartz. The parameters for fused silica and fused quartz are similar; it is understandable because both are amorphous. Our result,  $[dn_q/dT] = (8.49 \pm 0.05) \times 10^{-6} \text{K}^{-1}$  at 780 nm, is of the same order as those of the amorphous silica.

From the comparisons we can see that large discrepancies exist between the temperature dependences of refractive index of amorphous and crystalline quartz types; it is therefore worthwhile pointing out that the optical properties of different quartz types should be treated carefully. Wrong choice of the data will cause illogical result.

### 4.2. $[\partial n_q/\partial C]_T$

Figure 3 gives the measurement result of  $[\partial n_q/\partial C]_T$  at different temperatures. The data varied in a small range without a clear evidence to support the dependence of  $[\partial n_q/\partial C]_T$  on temperature. Fredericks *et al.* also pointed out that when the pH is lower than 5.5,  $[\partial n_q/\partial C]_T$  is nearly a constant [23]. We thus averaged all of the measured data for  $[\partial n_q/\partial C]_T$  and the obtained result was  $(2.16 \pm 0.41) \times 10^{-4} \text{ml}\cdot\text{mg}^{-1}$ .

Table 2 listed the results reported in the literatures at different conditions and our current result. From the table, we can see that the current result is very similar to Fredericks *et al.*'s work [23] and Hou *et al.*'s work [22], which is about  $2.0 \times 10^{-4} \text{ml}\cdot\text{mg}^{-1}$ , though the wavelengths are different from each other. In the protein crystal growth system, the pH, NaCl concentration and the buffer will affect the physical properties of the solution. However, it is very interesting to note that the concentration dependences of the refractive index of HEWL

solution are mostly similar to each other. It seems that the wavelength doesn't change  $[\partial n_s/\partial C]_T$  much. Other conditions including NaCl concentration, buffer, and pH all seemed not to have strong effect on  $[\partial n_s/\partial C]_T$ . From these facts we suggest that  $[\partial n_s/\partial C]_T$  is relatively insensitive to the change of wavelengths, NaCl concentration and pH.

#### 4.3. $[\partial n_s/\partial T]_C$

Fig. 4 gives the result of  $[\partial n_s/\partial T]_C$  at different concentrations. It is clear that  $[\partial n_s/\partial T]_C$  is dependent on the concentration. When the concentration increases, the absolute value of  $[\partial n_s/\partial T]_C$  increases. The data were fitted with a second order polynomial equation. The following empirical equation was obtained to predict the value of  $[\partial n_s/\partial T]_C$  at different concentrations:

$$\left[ \frac{\partial n_q}{\partial T} \right]_C = 2.477 \times 10^{-9} C^2 - 3.511 \times 10^{-7} C - 1.411 \times 10^{-4}. \quad (8)$$

The values of  $[\partial n_s/\partial T]_C$  at the concentration level 0 ~ 70 mg/ml were found to be in the range of  $-1.41 \times 10^{-4} \text{K}^{-1}$  ~  $-1.54 \times 10^{-4} \text{K}^{-1}$ .

There are few reports about the temperature dependence of the refractive index of HEWL solution in the literature except for Fredericks *et al.*'s work, which pointed out that the value varied in the range of  $-0.98 \times 10^{-4} \text{K}^{-1}$  ~  $-1.26 \times 10^{-4} \text{K}^{-1}$  at wavelength 578 nm, depending on the concentrations of NaCl and HEWL, and pH. In our case, the concentration level of NaCl and pH value were kept the same for all the experimental runs, thus the temperature dependence of the refractive index depended on the concentration level. However, we could not find a relationship match between Frederick *et al.*'s work and ours. However, the measurement result in the current study is of the same order with theirs.

The above data obtained have been proved applicable and reliable in the determination of HEWL concentration in the solution based on Eq. (3) [15, 34, 35]. The concentration can be measured at an accuracy up to  $\pm 0.36 \text{mg/ml}$  without applying phase shift method. If the phase-shift method can be utilized, it will further improve the accuracy. The above measurement data enable the determination of HEWL concentration not only at homogeneously constant temperature conditions, but also at various conditions when the temperature varies in the process, or even varies differently at different positions at the same time, thus making the concentration measurement method using interferometry more versatile and powerful. These parameters will also be applicable for other purpose where the temperature or concentration dependence of the refractive index of HEWL solution is necessary. However, for a measurement system using different wavelengths, similar dependences



determination should be carried out for accurate measurement. The method described in section 2 is also applicable for measurements under other conditions (different NaCl concentration, pH value, and buffer conditions, or even other solution systems).

## 5. Conclusions

In this paper, the temperature and concentration dependences of refractive index of HEWL solution, and the temperature dependence of the refractive index of quartz container were measured using a Mach-Zehnder interferometer. The measurement results are indispensable in the in-situ concentration measurement of HEWL solution using interferometry. The obtained data could also be useful for other purposes concerning the refractive index change versus temperature and/or concentration. The measurement technique used in this paper can be applied to other solution systems without taking any measures to determine the actual values of the refractive index of the solution.

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Figure captions:

Fig. 1. Schematic illustration of the studied cell configuration

Fig. 2. Schematic illustration of the experimental setups. (a) Mach-Zehnder interferometer and recording system; (b) Sample holder configuration. LD: laser diode; M1~M2: mirrors; Bs1~BS2: beam splitters; VCR: video cassette recorder.

Fig. 3. Dependence of refractive index of HEWL solution on concentration versus temperature

Fig. 4. Dependence of refractive index of HEWL solution on temperature versus concentration

Table 1. Temperature dependences of refractive index of different types of silica

Reference	Current work	[30]	[26]	[28]	[28]	[31]	[31]	[32]	[33]
$[dn_d/dT] (\times 10^{-6} K^{-1})$	8.49±0.05	12.46	6.20	-7.2	-8.8	-5.49	-6.53	8.66	8.5
Wavelength (nm)	780	780	632.8	632.8	632.8	643	643	632.8	587.6
What they called	Quartz container	Fused quartz	Fused quartz	Crystal quartz(O)	Crystal quartz(E)	Crystal quartz(O)	Crystal quartz(E)	Silica glass	Fused silica
Temperature (°C)	0~60	20~30	27~427	25~300	25~300	62	62	25~125	20

Table 2 Concentration dependences of the refractive index of HEWL solution at different conditions

Reference	Current work	[23]	[17,18]	[19, 20]	[22]	[24]
$[\partial n_s / \partial C]_T$ ( $\times 10^{-4} ml \cdot mg^{-1}$ )	2.16 ± 0.41	2.0 ± 0.1	1.73	~1.67	~2.0	1.86~1.88
Wavelength (nm)	780	578	632.8	547	532	632.8
NaCl (mg/ml)	40	8.7~29.2	~52.6	~40	~25	0.58~5.84
PH	4.60	4.5~5.5	4.50	5.5	4.5	7.4
Buffer	No buffer	0.05M NaOAc	0.05M NaOAc	0.1M NaOAc	0.05M NaOAc	0.01M HEPES

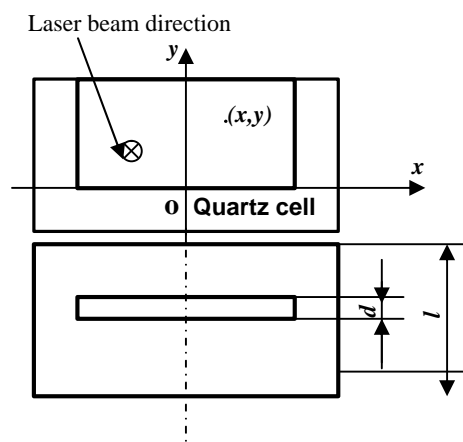
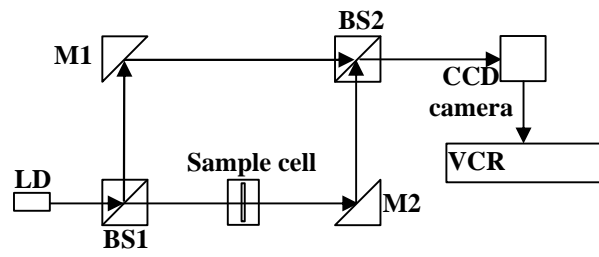
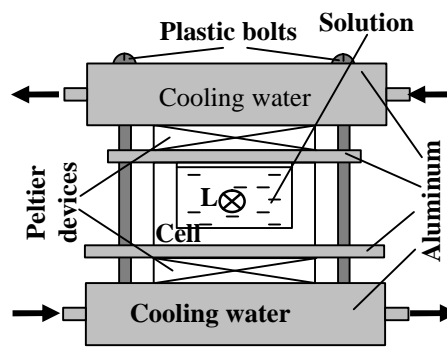


Fig. 1.



(a)



(b)

Fig. 2.

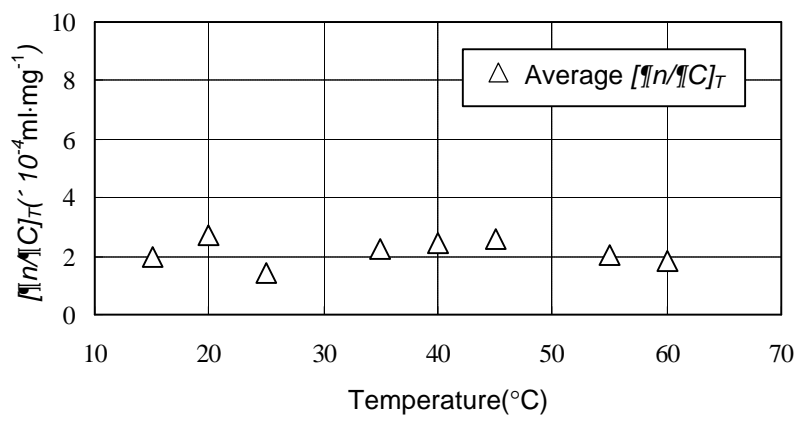


Fig. 3.

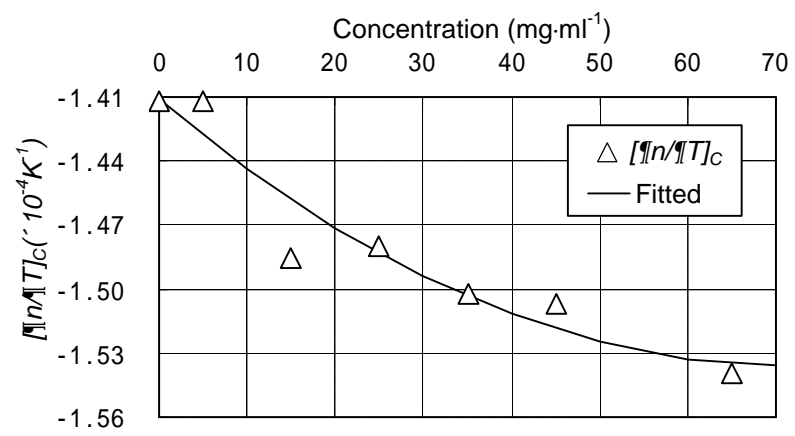


Fig. 4.