Visible light spectroscopic analysis of Methylene Blue in water; the resonance virtual equilibrium hypothesis

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ABSTRACT

After a critical approach to the centennial literature on Methylene Blue self-aggregation in water, accurate visible-light absorption spectra and thermodynamic equilibrium constants for the monomeric and dimeric forms of this dye in water at different temperatures were obtained by means of a novel procedure based on the Beer-Lambert equation. A thermo-solvatochromic effect was clearly detected in the monomer spectra, which we believe to be the main source of inaccuracy in all previous works. The only published explanation for this phenomenon, the existence of a colorless form of the monomer, is refuted here. Instead, a new notion has been introduced; the resonance virtual equilibrium hypothesis. According to this hypothesis the temperature-dependent absorption behavior of the monomer is provoked by the change in its electron charge distribution with the variation in the temperature-dependent dielectric constant of water. The monomer charge distribution stands between those of the virtual resonance forms, being the absorption spectrum of the monomer a composition of the theoretical spectra for the virtual mesomers, whose proportion is established by a temperature-dependent equilibrium constant. The application of this hypothesis allowed the virtual equilibrium constants and spectra for the mesomers to be evaluated with remarkable accuracy.

KEYWORDS: *Methylene Blue; Uv-Visible spectrophotometry; equilibrium constant; thermo-solvatochromism; self-aggregation; resonance form*

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1. INTRODUCTION

Concentration-dependent metachromasy of aqueous Methylene Blue (MB) solutions is well known and can be related to the formation of aggregates [1-4]. At low MB concentration (below ~20 ppm, according to some authors [5], or ~70 ppm, according to others [6]) the existence of aggregation beyond the dimeric form of the dye (i.e. trimeric or tetrameric forms) can be neglected. Within this concentration range, object of the present work, the specific molar absorptivity of the dimer affects the shape of the Uv-Vis spectra according to its ratio in the dimer/monomer mixture. The commonly accepted existence of this effect is only challenged by the group of González-Aguilar [7], although their calculations have been questioned in [8]. In aqueous MB solutions, monomer molecules and dimers coexist at ambient temperature, both showing fairly different absorption spectra [9, 10]. The concentration of the dimeric form increases with the total concentration of MB molecules due to the specific form of the reaction constant for the monomer/dimer equilibrium. Such equilibrium has been studied since long and a variety of thermodynamic (entropy and enthalpy of dimerization) and optical (molar attenuation coefficients) data are available in literature [1, 6, 9, 11-18]. These data were obtained by means of a methodology firstly applied by Rabinowitch and Epstein [11], which consists of solving the equations that link the absorbance with the monomer and dimer concentrations at two or several wavelengths to derive the molar attenuation coefficients for the monomer and dimer, either via extrapolation techniques [1, 6, 12-14] or by the error minimization [9, 15]. However, in spite of the long time that the scientific community has dedicated to this subject, the equilibrium constants and optical parameters have always been evaluated with a high degree of uncertainty (Table 1), which makes us believe that the cause of such inaccuracy has escaped the attention of the scientists to date. The present work proves that the reason for the uncertainty lies in a common and erroneous assumption since the time of Rabinowitch and Epstein [11] for the calculation of the aggregation degree and the dimerization

constant. This assumption is that the absorption spectrum of the monomer is independent of the temperature (*invariant-monomer assumption* from now on). However, this universally employed assumption is in contradiction with the long known fact that the maximum attenuation coefficient of the monomer spectrum (obtained via extrapolation at a very low MB concentration) shows a slight decrease at increasing temperatures [12] (see Table 1). The authors that detected this trend, Lewis *et al.* [12], proposed as an explanation the existence in solution of a “considerable” amount of a colorless form of the MB monomer. According to them, this form could be produced by the hydrolytic addition of water to the cationic monomer, process that they found surprisingly favored at increasing temperatures (the colorless species might be similar to the Leuco-Methylene Blue molecule, which is formed by the reduction of monomeric MB in the absence of oxygen [19]). To our knowledge, no more studies about the effect of temperature on the light absorption by the MB monomer in water are available in literature.

In the present work we have employed in a novel manner the old and simple equations used by most authors for nearly a century, with two main objectives; (i) to evaluate reliable and accurate optical and thermodynamic parameters for MB in water solutions at low concentration, and (ii) to shed fresh light on the spectroscopic behavior of the MB monomer in water at varying temperatures, that calls into question not only the *invariant-monomer assumption* but also the hypothesis by Lewis *et al.* [12].

2. EXPERIMENTAL

The absorption spectra (400–800 nm at 1 nm step) of 6 MB (C.I. 52015; analytical grade) solutions in deionized water in the 1.1×10^{-6} – 3.4×10^{-5} mol L⁻¹ (0.35–11 ppm) concentration range were obtained at temperatures in the 9–70°C range using an Uv-Vis spectrometer (Shimadzu UV-2401PC) with an Uv quartz cuvette of 700 µL volume and 1 cm path length. The temperature of the cell was kept constant

using a Lauda Alpha RA8 thermo-circulating bath. Every measure was repeated thrice. Ad hoc Microsoft® Excel macros were designed to perform the error minimization tasks, incorporating the Solver complement of Microsoft® Excel configured to employ the Generalized Reduced Gradient algorithm with central derivatives.

3. HISTORICAL BACKGROUND

Application of the Beer-Lambert equation to the absorption spectrum of Methylene Blue in water at a given wavelength yields:

$$A_{\lambda} = \varepsilon_{\lambda} L C_{MB} = A_{\lambda}^m + A_{\lambda}^d = \varepsilon_{\lambda}^m L C_m + \varepsilon_{\lambda}^d L C_d \quad (1)$$

where A_{λ} is the absorbance at the λ wavelength, ε_{λ} , ε_{λ}^m and ε_{λ}^d are the molar attenuation coefficients of the solution, the monomer and the dimer, respectively, at the same wavelength ($\text{L mol}^{-1} \text{cm}^{-1}$), L is the path length (cm) and C_{MB} , C_m and C_d are the MB, monomer and dimer concentrations, respectively (mol L^{-1}). The total MB concentration can be evaluated from C_m and C_d as:

$$C_{MB} = C_m + 2C_d \quad (2)$$

The objective of all calculation methods has been to evaluate the molar attenuation coefficients for the monomer and the dimer, from which the aggregation degree can be readily calculated. In the pioneering work by Rabinowitch and Epstein [11], the molar attenuation coefficients for the MB dimer and monomer at two wavelengths (656.5 and 600 nm) were evaluated by extrapolation to $C_{MB}=0$ and $C_{MB}=\infty$ of the observed molar attenuation coefficients. Then the fraction of monomer was calculated by solving the following equation

$$X_m = \frac{C_m}{C_{MB}} = \frac{\varepsilon_{\lambda} - 0.5 \varepsilon_{\lambda}^d}{\varepsilon_{\lambda}^m - 0.5 \varepsilon_{\lambda}^d} \quad (3)$$

where ε_λ is the observed **molar** attenuation coefficient at λ . This equation directly derives from equation (1), and the value of K_d can be subsequently obtained as

$$K_d = \frac{C_d}{C_m^2} = \frac{1-X_m}{2C_{MB}X_m^2} \quad (4)$$

The values of the so obtained **molar** attenuation coefficients are indicated in Table 1, together with those obtained by other authors and, when available, the derived thermodynamic parameters. Bergmann and O’Konski [6] turned the method of Rabinowitch and Epstein upside down by firstly assuming a certain value of K_d from which they evaluated X_m at different values of C_{MB} via equation (4). Then, they represented the values of X_m versus the observed molar attenuation coefficients and obtained the values of ε_λ^m and ε_λ^d by extrapolation at $X_m=1$ and $X_m=0$, respectively [equation (3)]. The value of K_d was varied until attaining coefficients of determination for equation (4) closest to 1 for several λ values. Although the **molar** attenuation coefficients could be determined by this method, the authors themselves acknowledged that it was not sensitive enough to distinguish between several equilibrium constants. Gosh [13] used the Rabinowitch and Epstein method but with the condition that the **molar** attenuation coefficients of all the aggregates had to be the same at the wavelength for maximum absorbance in the monomer spectrum, assumption that can in no way be justified. Another typical extrapolation technique is to assume that $C_{MB} \approx C_m$ and $C_{MB} \approx C_d$ at a low and a at a high dye concentration, respectively [12]. This assumption may work fairly well for the monomer attenuation coefficients, but it is inadequate for the dimer, since a high increase in the MB concentration also produces increasingly complex polymers [1].

The extrapolation methods produce large errors in the degree of association from only small differences in the **molar** attenuation coefficients [1] and the evaluation of the **molar** attenuation coefficient for the dimer is especially subjected to error [11]. With the advent of modern computers, the error minimization

methods were introduced in an attempt to solve this problem [9, 15]. Spencer and Sutter [15] combined equations (1), (2) and (4) to obtain the following equation:

$$A_{\lambda} = \left(\varepsilon_{\lambda}^m - \frac{\varepsilon_{\lambda}^d}{2} \right) L \frac{\sqrt{1+8K_d C_{MB}} - 1}{4K_d} + \varepsilon_{\lambda}^d L \frac{C_{MB}}{2} \quad (5)$$

Note that equation (5) here is different from equation (3) in [15], probably due to a transcription error in the work referred to. The minimization of the sum of squared deviations of experimental from calculated [by, hopefully, equation (5)] absorbances gave the values for the parameters at 610 and 660 nm indicated in Table 1. As can be seen in the table, some of the parameters evaluated by this method ($\varepsilon_{\lambda_1}^d$, K_d , ΔH_d and ΔS_d) suffer from a high dispersion (especially $\varepsilon_{\lambda_1}^d$), which makes it difficult to have an accurate evaluation of the aggregation degree. Furthermore, contrary to their own claim, the values of ΔH_d and ΔS_d (enthalpy and entropy of dimerization) obtained by the authors are much higher (in absolute values) than those obtained by a variety of investigators (Table 1). Yazdani *et al.* [9] employed a more complex methodology based on the construction of matrices including the absorbances in the whole wavelength range at different temperatures and MB concentrations. With this technique, the dimer spectrum, the monomer concentration profile and the equilibrium constant were evaluated by imposing three constrains; (i) the spectrum of the monomer, which is measured separately, (ii) the total concentration of the dye [equation (2)] and (iii) the thermodynamic relation between the temperature and the equilibrium constant (van't Hoff equation). As in [15], the sum of squared differences between experimental and predicted absorbances was used as a goodness of fit criterion. In spite of the complexity of the model, the results were far from being free of dispersion, as can be observed in Table 1 (see footnotes), and the dimer contribution to the characteristic peak of the monomer ($\varepsilon_{\lambda_1}^d$ in Table 1), evaluated from Figure 3-1b in [9], resulted to be much higher than that found by the rest of authors. In

the following section a new error minimization method will be proposed. In spite of the simplicity of the method, it allows the optical and thermodynamic parameters to be accurately evaluated.

4. METHOD FOR LOW MB CONCENTRATION

At low MB concentrations (below ~20 ppm, according to some authors [5], or ~70 ppm, according to others [6]) the existence of the trimeric or tetrameric forms of the dye in solution can be neglected. Under this circumstance, the method proposed here simply consists of using the Beer-Lambert equation at two different wavelengths of the spectrum (λ_1 and λ_2):

$$A_{\lambda_1} = \varepsilon_{\lambda_1} LC_{MB} = A_{\lambda_1}^m + A_{\lambda_1}^d = \varepsilon_{\lambda_1}^m LC_m + \varepsilon_{\lambda_1}^d LC_d \quad (6)$$

$$A_{\lambda_2} = \varepsilon_{\lambda_2} LC_{MB} = A_{\lambda_2}^m + A_{\lambda_2}^d = \varepsilon_{\lambda_2}^m LC_m + \varepsilon_{\lambda_2}^d LC_d \quad (7)$$

In these equations, the molar attenuation coefficients are independent of temperature under the *invariant-monomer assumption*. It will be proven in the *Results and discussion* section that this assumption must be qualified. λ_1 and λ_2 should be close to the wavelengths at maximum absorbance for the monomer and the dimer, 664 and 610 nm, respectively [9]. From equations (2), (6) and (7) the following equations are easily obtained:

$$C_{MB} = \alpha_1 A_{\lambda_1} + \alpha_2 A_{\lambda_2} \quad (8)$$

$$C_m = \beta_1 A_{\lambda_1} + \beta_2 A_{\lambda_2} \quad (9)$$

in which α_1 , α_2 , β_1 and β_2 (mol L^{-1}) are defined as:

$$\alpha_1 = \frac{-2\varepsilon_{\lambda_2}^m + \varepsilon_{\lambda_2}^d}{(\varepsilon_{\lambda_1}^m \varepsilon_{\lambda_2}^d - \varepsilon_{\lambda_2}^m \varepsilon_{\lambda_1}^d)L} \quad (10)$$

$$\alpha_2 = \frac{2\varepsilon_{\lambda_1}^m - \varepsilon_{\lambda_1}^d}{(\varepsilon_{\lambda_1}^m \varepsilon_{\lambda_2}^d - \varepsilon_{\lambda_2}^m \varepsilon_{\lambda_1}^d)L} \quad (11)$$

$$\beta_1 = \frac{\varepsilon_{\lambda_2}^d}{(\varepsilon_{\lambda_1}^m \varepsilon_{\lambda_2}^d - \varepsilon_{\lambda_2}^m \varepsilon_{\lambda_1}^d)L} \quad (12)$$

$$\beta_2 = \frac{-\varepsilon_{\lambda_1}^d}{(\varepsilon_{\lambda_1}^m \varepsilon_{\lambda_2}^d - \varepsilon_{\lambda_2}^m \varepsilon_{\lambda_1}^d)L} \quad (13)$$

From these parameters, the molar attenuation coefficients can be evaluated as:

$$\varepsilon_{\lambda_1}^m = \frac{\beta_2 - \alpha_2}{(\alpha_1 \beta_2 - \alpha_2 \beta_1)L} \quad (14)$$

$$\varepsilon_{\lambda_2}^m = \frac{\alpha_1 - \beta_1}{(\alpha_1 \beta_2 - \alpha_2 \beta_1)L} \quad (15)$$

$$\varepsilon_{\lambda_1}^d = \frac{-2\beta_1}{(\alpha_1 \beta_2 - \alpha_2 \beta_1)L} \quad (16)$$

$$\varepsilon_{\lambda_2}^d = \frac{2\beta_2}{(\alpha_1 \beta_2 - \alpha_2 \beta_1)L} \quad (17)$$

Equations (10) and (11) resemble the elements of the inverse $\mathbf{R}$ rotation matrix equation by Yazdani *et al.* [9], this being the only similarity with the present method, which, as will be seen, yields optical and thermodynamic values that differ greatly from those obtained by these authors. C_{MB} can be also expressed according to the following equation, derived from equations (2), (4) and (9):

$$C_{MB} = (\beta_1 A_{\lambda_1} + \beta_2 A_{\lambda_2})[1 + 2K_d(T) \times (\beta_1 A_{\lambda_1} + \beta_2 A_{\lambda_2})] \quad (18)$$

Constant K_d (L mol^{-1}) may be expressed either as a function of A_{λ_1} and A_{λ_2} or as a function of the temperature (van't Hoff equation):

$$K_d(A_\lambda) = \frac{C_{MB} - (\beta_1 A_{\lambda_1} + \beta_2 A_{\lambda_2})}{2 \times (\beta_1 A_{\lambda_1} + \beta_2 A_{\lambda_2})^2} \quad (19)$$

$$K_d(T) = e^{\left(\frac{\Delta S_d}{R} - \frac{\Delta H_d}{RT}\right)} \quad (20)$$

where ΔS_d and ΔH_d are the enthalpy and entropy of dimerization, respectively. In order to determine the values of α_1 , α_2 , β_1 and β_2 and the thermodynamic parameters [ΔS_d and ΔH_d], the following mean square error function can be minimized for the $N \times M$ values of A_{λ_1} and A_{λ_2} obtained at different dye concentrations and temperatures:

$$E_T = \frac{1}{N \times M} \sum \{C_{MB} - (\alpha_1(T)A_{\lambda_1} + \alpha_2(T)A_{\lambda_2})\}^2 + \frac{1}{N \times M} \sum \{C_{MB} - (\beta_1(T)A_{\lambda_1} + \beta_2(T)A_{\lambda_2})[1 + 2K_d(T) \times (\beta_1(T)A_{\lambda_1} + \beta_2(T)A_{\lambda_2})]\}^2 \quad (21)$$

As already commented, under the *invariant monomer assumption* α_1 , α_2 , β_1 and β_2 parameters should be independent of the temperature [eqs. (10) to (13)]. However, as will be proven in the *Results and discussion* section, a clear temperature-dependence is observed in these parameters, as result of which they are expressed as functions of the temperature in equation (21). It has been also noticed that slight variations in the values of $\alpha_1(T)$, $\alpha_2(T)$, $\beta_1(T)$ and $\beta_2(T)$ can produce a severe variation in some of the optical parameters evaluated from equations (14) to (17). In order to circumvent this issue, the following procedure for the determination of the molar attenuation coefficients has been employed. A set of molar attenuation coefficients together with initial values of ΔS_d and ΔH_d (*i.e.* some of those indicated in Table 1 for MB) are used as initial guesses for the error minimization process with the Solver tool in order to minimize the total error E_T [for which $\alpha_1(T)$, $\alpha_2(T)$, $\beta_1(T)$ and $\beta_2(T)$ parameters are calculated via equations (10) to (13)] under the constrain of positive values of the molar attenuation coefficients. In this way, convergence to very low error values is rapidly attained, and precise values of the molar attenuation coefficients and thermodynamic parameters are finally obtained. The goodness of fit is categorized by both the values of the error E_T and the deviation of $K_d(T)$ from $K_d(A_\lambda)$ at the different conditions.

A chief difference with respect to previous error minimization methods is the form of the error function which rely on experimental and predicted concentration differences [equations (8) and (18)], instead of the common procedure of minimizing the error in absorbance (in [20] both the concentration and absorbance differences are minimized).

For higher MB concentrations (*i.e.* over 70 ppm [1]), the presence of higher aggregates (*i.e.* trimers and tetramers) must be accounted for. A more comprehensive method, applicable to higher dye concentrations, will be presented in the near future.

5. RESULTS AND DISCUSSION

Figure 1 shows a typical absorption spectrum of MB in the 400–800 nm wavelength range. Linear baselines were automatically evaluated for each spectrum from the minimum absorbance in the 400–450 nm range to the minimum absorbance in the 710–800 nm range. The values of absorbance at 664 (A_{λ_1}) and 610 nm (A_{λ_2}) were thus obtained after the baseline correction (Fig. 1). The inset in Fig. 1 shows the variation of the A_{664}/A_{610} ratio for the different MB concentrations and temperatures analyzed. The trends are a clear proof of the dependence of the monomer/dimer ratio on MB concentration and temperature.

5.1. Optical and thermodynamic parameters. Refuting the *invariant-monomer assumption*

Values of ε_{664}^m , ε_{610}^m , ε_{664}^d , ε_{610}^d , ΔS_d and ΔH_d were evaluated via minimization of the E_T error following the procedure above described. The results are indicated in Table 1. The molar attenuation coefficients for the monomer show an appreciable decrease with the increase of temperature for $\lambda_1=664$ nm (from $\sim 8 \times 10^4$ L mol⁻¹ cm⁻¹ at 9°C to $\sim 7 \times 10^4$ L mol⁻¹ cm⁻¹ at 60°C) whereas the monomer attenuation

coefficients at 610 nm show a minute variation and the **molar** attenuation coefficients for the dimer are invariant regardless the temperature. These values permit the minimization of the E_T error to an extremely low value ($E_T=1.6\times10^{-14}$). The low standard deviations of the K_d values at the different temperatures can be observed in Table 1 and in the error bars of $K_d(A_\lambda)$ included in Figure 2. Also the low E_T error may be visualized in the remarkable coincidence of the $K_d(A_\lambda)$ data with the $K_d(T)$ curve (Fig. 2). As expected, the entropy change is negative due to the association of two similarly charged species [21]. The fraction of monomer can be evaluated at the different conditions by means of the following equation:

$$X_m = \frac{c_m}{c_{MB}} = \frac{\beta_1 A_{\lambda_1} + \beta_2 A_{\lambda_2}}{c_{MB}} \quad (22)$$

Figure 3 shows the values of monomer fraction obtained for the different MB concentrations and temperatures. Unsurprisingly, the monomer molar fraction increases with the temperature [9] and with the decrease of MB concentration (equilibrium constant). Finally, the dimer and monomer spectra in the 500–695 nm range were obtained by a simple procedure. The wavelength range was divided into two sectors; at $\lambda > 630$ nm, λ_1 was varied between 631 and 695 nm and λ_2 was fixed at 610 nm, whereas at $\lambda \leq 630$ nm, λ_1 was fixed at 664 nm and λ_2 was varied between 500 and 630 nm. Then, the E_T error was minimized for the different (λ_1, λ_2) couples while keeping the molar attenuation coefficients at 610 and 664 nm and the thermodynamic parameters (ΔS_d and ΔH_d) equal to those previously obtained (Table 1). The results of applying this procedure are shown in Figure 4. The upper plot of the figure includes the $K_d(A_\lambda)$ and $K_d(T)$ values together with the E_T value. As can be observed, both the dimeric constant deviations and the errors are very low within almost the whole wavelength range, except in the zone over ~ 680 nm, where the steep absorbance decrease (Fig. 1) may allow an appreciable variation in the absorbance to be produced by even the slightest deviation in the wavelength. This issue will be further

analyzed in the last part of this discussion. The spectrum obtained for the dimer displays a profile which greatly differs from those attained by other authors [6, 9]. It is conspicuous the absence of the maxima at ~664 nm obtained by Yazdani *et al.* [9] and at ~695 nm by Bergmann and O’Konski [6]. Only a maximum at 606 nm (common to other works) and a decaying shoulder from ~650 nm are observed in the figure. However, the most striking result appreciated in Figure 4 is the temperature dependence of the monomer spectrum, with a maximum absorbance that decreases at increasing temperatures (Table 1), showing in parallel a small blueshift (from 664 nm at 9°C to 662 nm at 60°C). This fact proves that the *invariant-monomer assumption* is incorrect. We believe that this implicit assumption in all previous works is the main source of inaccuracy in both the optical and thermodynamic parameters for MB self-aggregation in water reported in literature (Table 1).

5.2. Refuting the colorless-monomer assumption

As mentioned in the Introduction section, Lewis *et al.* [12] found the same effect in MB spectra obtained at a very low concentration (Table 1 and Figure 5A). The diminution observed in the maximum molar attenuation coefficient with the temperature was attributed to the existence of an additional MB monomer that was assumed to be colorless [12] (*colorless-monomer assumption* from now on). This species might be the known Leuco-Methylene Blue molecule, which is formed by the reduction of monomeric MB in the absence of oxygen [19], presenting an absorbance maximum at ~351 nm, as predicted by TD-DFT calculations [22]. Under the *colorless-monomer assumption*, the total MB concentration must be evaluated as:

$$C_{MB} = C_m + C_{clm} + 2C_d \quad (23)$$

where C_{clm} stands for the colorless monomer concentration (mol L⁻¹). According to Lewis *et al.* [12] the colorless monomer is formed via the amphoteric addition of water to the colorful monomer. Thus, the following equilibrium constant, K_{clm} , can be proposed:

$$K_{clm} = \frac{C_{clm}}{C_m} \quad (24)$$

This equation results from the obvious consideration that the concentration of water remains essentially unchanged at low C_{MB} values. At a very low MB concentration, the dimer is practically absent ($X_m \approx 1$; Fig. 3) and the Beer-Lambert equation at a λ wavelength can be expressed as:

$$A_\lambda = A_\lambda^m = \varepsilon_\lambda^m L C_m = \frac{\varepsilon_\lambda^m}{1+K_{clm}} L C_{MB} \quad (25)$$

Therefore, the observed temperature-dependent molar attenuation coefficients in the absence of dimer can be expressed as:

$$\varepsilon_\lambda^m(T) = \frac{\varepsilon_\lambda^m}{1+K_{clm}} \quad (26)$$

where $\lambda=660$ nm in the work by Lewis *et al.* [12]. The data shown in Figure 5A are, in principle, consistent with the above equation, provided the K_{clm} constant increases with the temperature. Lewis *et al.* [12] found this trend remarkable, but they still reasoned that it might be possible. On the other hand, as deduced from equation (26), one condition must be accomplished: when the formation of aggregates is discarded, the trend of the temperature-dependent molar attenuation coefficients with the temperature must be a decreasing one *for all wavelengths*. This condition was checked with different molar attenuation coefficients obtained in this work from the spectra at the lowest concentration ($C_{MB} = 1.1 \times 10^{-6}$ mol L⁻¹; $X_m > 0.95$; Fig. 3), and it was found that, in many cases, the temperature-dependent coefficients either increased with the temperature or remained more or less constant. Examples of opposite trends are shown in Figure 5B, for wavelengths in the interval 650–690 nm, within which the

already insignificant effect of the residual dimer concentration is reduced even more (Fig. 4). Therefore, *the colorless-monomer assumption* is also invalid to explain the different monomer absorption spectra of Figure 4.

Two other alternatives that may explain the temperature-dependent absorption behavior observed in Figures 4 and 5 must be discarded. (i) The little significant MB monomer adsorption on the quartz walls of the cuvette [23] cannot be claimed to be the cause of this behavior, since it would produce the opposite effect; the lower the temperature the higher the MB monomer adsorption and, consequently, the lower the light absorption. (ii) The presence of a mixture of two or more different colorful MB monomers is out of question. The potential presence of a protonated MB monomer, in which the proton is attacking the electron-rich heterocyclic nitrogen [22], would produce a bathochromic effect with the appearance of a peak at ~745 nm [24], which is absent in all of the spectra obtained in this work. Furthermore, this protonated monomer would be in a significant amount only at very acidic conditions [25]. The blueshift observed on increasing the temperature is certainly too small to be associated to the formation of Bernthsen's methylene violet, the main product from the hydroxyl attack to MB (absorbance maximum at 520 nm in toluene), which is only produced at basic conditions (pH=13) [22]. Besides, different colorful monomers would potentially produce a variety of dimeric forms with different absorption spectra. In the present work only one absorption spectrum for the dimeric form was needed to perform an accurate fitting to all the absorption profiles.

Therefore, the temperature-dependent absorption behavior of the monomeric form must be ascribed to the presence of a single MB monomer whose molar attenuation coefficient *actually changes* with the temperature. The following hypothesis raises an explanation about how this can occur.

5.3. The resonance virtual equilibrium hypothesis

The cationic MB molecule is a resonance hybrid with an electronic charge distribution somewhere in between those of the two virtual molecules shown in Figure 6 [22, 26]. The *mesomer I* has a positive charge on the heterocyclic sulphur (the heterocyclic nitrogen is known to bear a negative charge in the ground state [22]), whereas the *mesomer II* bears the positive charge on the exocyclic nitrogen atom. Figure 6 also shows the direction and values of the theoretical dipole moments in the ground state and in the excited singlet state (μ_G and μ_E , respectively) for both mesomers, as reported by Párcányi *et al.* [27]. The experimental dipole moments in the ground and excited singlet states ($\mu_{G,EXP}$ and $\mu_{E,EXP}$, respectively) have been also evaluated by different methods and are available in the literature [27, 28]. The values indicated in Figure 6 [28] certify a large dipole moment change during transition from the ground state to the excited singlet state, which is due to intermolecular charge transfer and resonance structure contribution. The experimental dipole moment mean value in the excited singlet state (5.0 D) is in good agreement with the theoretical value (4.7 D) calculated with a positive charge on the exocyclic nitrogen. As suggested in [27] this is an indication of a change of distribution of charges in electronically excited states, predicting a migration of the positive charge from the ring sulfur atom to the exocyclic nitrogen atom in the excited singlet state of MB.

The intramolecular charge transfer in the MB molecule is sensitive to the solvent, *i.e.*, it shows solvatochromism [28-30]. However, contrary to the typical betaine-type probes employed in solvatochromic studies [31, 32], the solvatochromism of MB is poor, that is to say, the solvent polarity has no regular effect on its spectral behavior [28]. In spite of this, Gilani *et al.* [28] reported a rather obvious bathochromic shift in the MB absorption spectra for increasing values of solvent dipolarity/polarizability (π^*), trend that was also reported in a previous work for all phenothiazines, such as MB, with the exception of thionine [27]. In principle, this fact is consistent with the small

blueshift observed in Figure 4 at increasing temperatures, since the dielectric constant of water is known to decrease with temperature, from 83.8 at 10°C to 63.8 at 70°C [33, 34]. Thus, the observed blueshift can be considered as a type of thermo-solvatochromic effect [35, 36]. This picture is, however, rather simplistic and does not explain the opposite trends observed in Figure 5B. We believe that the different monomer spectra shown in Figure 4 should not be explained only through thermo-solvatochromic shifts at the wavelength for maximum absorbance, but as the result of the convolution of two fixed absorption spectra whose proportion varies with temperature. And this fact must be reconciled with the existence of a single monomer, that is to say, two spectra for one monomer. To make this possible the *resonance virtual equilibrium hypothesis* is proposed hereafter. The hypothesis affirms that the temperature-dependent absorption behavior of the monomer is a consequence of a thermo-solvatochromic effect, provoked by the change in the electron charge distribution in the monomer with the variation in the temperature-dependent dielectric constant of water. Within this frame, the main assumptions of the hypothesis are (i) the electron charge distribution of the monomer at a given temperature stands between those of the virtual resonance forms (Figure 6), and (ii) the absorption spectrum of the monomer is a composition of the theoretical spectra for the virtual resonance forms, whose proportion is established by a temperature-dependent equilibrium constant (*additivity assumption*). Regarding the first assumption, it is known from computational studies that passing from isolated MB to aqueous (hydrated) MB implies an electronic density transfer in the longitudinal direction from the more external atoms (those bonded to the extremity rings) to the atoms of the central ring of the molecules [37], meaning that the polar environment leads to the stabilization of structures that move away from *mesomer I* towards *mesomer II*. Besides, Párcányi *et al.* [27] indicated that the excited singlet state, which, as commented above, resembles more the *mesomer II* structure, was more stabilized when the solvent polarity increased. These facts permit to assume that the charge distribution in the MB

monomer varies in the direction from *mesomer II* to *mesomer I* as the temperature increases or, to put it another way, as the water polarity decreases. With respect to the linearity concept implied in the *additivity assumption*, this is not dissimilar to that made to build the empirical solvent polarity scales [28-30], but in this work it comprises the spectrum in the whole wavelength range analyzed, and not only the wavelength at the maximum absorbance. In any case, the *additivity assumption* will be either validated or refuted depending on the goodness degree of the fitting procedures performed hereafter.

The *virtual* equilibrium between mesomeric forms can be defined by the following equilibrium constant:

$$K_m = \frac{C_{mI}}{C_{mII}} \quad (27)$$

in which C_{mI} and C_{mII} must be viewed as the contributions of the charge distributions of *mesomers I* and *II*, respectively, to the charge distribution of the actual resonance hybrid (*additivity assumption*), and not as factual concentrations. The K_m equilibrium constant is evaluated as (van't Hoff equation):

$$K_m(T) = e^{\left(\frac{\Delta S_m}{R} - \frac{\Delta H_m}{RT}\right)} \quad (28)$$

Thus, the monomer concentration can be evaluated as:

$$C_m = C_{mI} + C_{mII} = C_{mII} \times (1 + K_m) \quad (29)$$

Therefore, following the *additivity assumption*, at very low MB concentrations equation (25) can be rewritten as:

$$A_\lambda = A_\lambda^m = \varepsilon_\lambda^m LC_m = \frac{\varepsilon_\lambda^{mII} + \varepsilon_\lambda^{mI} K_m}{1 + K_m} LC_{MB} \quad (30)$$

where ε_λ^{mI} and $\varepsilon_\lambda^{mII}$ are the theoretical molar attenuation coefficients of *mesomers I* and *II*, respectively.

In this case the observed temperature-dependent molar attenuation coefficients can be expressed as:

$$\varepsilon_{\lambda}^m(T) = \frac{\varepsilon_{\lambda}^{mII} + \varepsilon_{\lambda}^{mI} K_m}{1 + K_m} \quad (31)$$

where $\lambda=660$ nm in the work by Lewis *et al.* [12] (Figure 5A). Equations (28) and (31) were fitted to the spectra at the lowest concentration ($C_{MB} = 1.1 \times 10^{-6}$ mol L⁻¹; $X_m > 0.95$; Fig. 3); both the theoretical molar attenuation coefficients for the resonance structures, $\varepsilon_{\lambda}^{mI}$ and $\varepsilon_{\lambda}^{mII}$, and the thermodynamic parameters (ΔS_m and ΔH_m) were obtained by minimizing the square differences of the calculated and the experimental values of $\varepsilon_{\lambda}^m(T)$. This procedure yielded values for ΔS_m and ΔH_m of 24.0 J mol⁻¹ K⁻¹ and 8.1 kJ mol⁻¹, respectively (Table 2). According to the *additivity assumption*, the C_{mI}/C_{mII} ratios obtained with the virtual equilibrium constant allow the charge distribution in the monomer to be placed in specific points within the limits established by both resonance forms at the different temperatures; thus, C_{mI}/C_{mII} varies from 0.58 at 9°C to 0.98 at 60°C, meaning that, in the whole temperature range studied, the charge distribution in the monomer is closer to that of *mesomer II* than to that of *mesomer I*. Figure 7A shows the calculated absorption spectra for both mesomers ($\varepsilon_{\lambda}^{mI}$ and $\varepsilon_{\lambda}^{mII}$), whereas Figure 7B reproduces the calculated and experimental spectra at the different temperatures [$\varepsilon_{\lambda}^m(T)$] for the analyses performed at the lowest MB concentration. For the sake of clarity, Figures 7C to 7E show augmented areas of Figure 7B. The agreement between calculated and experimental plots must be considered remarkable. The continuous curves in Figures 5A and 5B represent the best equation (31) fits to the data reported by Lewis *et al.* [12] and to the data obtained in this work, respectively, performed in both cases with the same thermodynamic parameters (Table 2). The opposite trends shown in Figure 5B are now reasonably reproduced thanks to the resonance virtual equilibrium hypothesis via equation (31). The values of ε_{660}^{mII} and ε_{660}^{mI} evaluated with this equation are 11.0×10^4 and 4.6×10^4 L mol⁻¹ cm⁻¹ for the data by Lewis *et al.* [12] (Figure 5A), and 9.2×10^4 and 5.0×10^4 L mol⁻¹ cm⁻¹ for the data obtained here (Figure 7A), respectively. The similarity of values is rather acceptable, especially if the following

sources of uncertainty, which do not affect the values of the thermodynamic parameters, are taken into account: (i) the wavelength accuracy: for instance a small increment in the wavelength would imply a higher ε_{660}^{mII} value and a lower ε_{660}^{mI} value (Fig. 7A), and (ii) the concentration error: low MB concentrations (<0.35 ppm), that involve smaller precision in the MB weighing or a higher number of dilutions, are more likely to introduce errors in the experimental molar attenuation coefficients than higher MB concentrations. This is why more accurate mesomers' spectra must be re-evaluated from the absorption curves for all the MB concentrations and temperatures, task that can be undertaken by introducing small changes in some of the equations of the method. Under the resonance virtual equilibrium hypothesis equations (10) to (13), (18) and (21) become:

$$\alpha_1 = \frac{-2\varepsilon_{\lambda_2}^{mII} + \varepsilon_{\lambda_2}^d + (\varepsilon_{\lambda_2}^d - 2\varepsilon_{\lambda_2}^{mI}) \times K_m}{[(\varepsilon_{\lambda_1}^{mII} \varepsilon_{\lambda_2}^d - \varepsilon_{\lambda_2}^{mII} \varepsilon_{\lambda_1}^d) + (\varepsilon_{\lambda_1}^{mI} \varepsilon_{\lambda_2}^d - \varepsilon_{\lambda_2}^{mI} \varepsilon_{\lambda_1}^d) \times K_m] L} \quad (32)$$

$$\alpha_2 = \frac{2\varepsilon_{\lambda_1}^{mII} - \varepsilon_{\lambda_1}^d - (\varepsilon_{\lambda_1}^d - 2\varepsilon_{\lambda_1}^{mI}) \times K_m}{[(\varepsilon_{\lambda_1}^{mII} \varepsilon_{\lambda_2}^d - \varepsilon_{\lambda_2}^{mII} \varepsilon_{\lambda_1}^d) + (\varepsilon_{\lambda_1}^{mI} \varepsilon_{\lambda_2}^d - \varepsilon_{\lambda_2}^{mI} \varepsilon_{\lambda_1}^d) \times K_m] L} \quad (33)$$

$$\beta_1 = \frac{\varepsilon_{\lambda_2}^d}{[(\varepsilon_{\lambda_1}^{mII} \varepsilon_{\lambda_2}^d - \varepsilon_{\lambda_2}^{mII} \varepsilon_{\lambda_1}^d) + (\varepsilon_{\lambda_1}^{mI} \varepsilon_{\lambda_2}^d - \varepsilon_{\lambda_2}^{mI} \varepsilon_{\lambda_1}^d) \times K_m] L} \quad (34)$$

$$\beta_2 = \frac{-\varepsilon_{\lambda_1}^d}{[(\varepsilon_{\lambda_1}^{mII} \varepsilon_{\lambda_2}^d - \varepsilon_{\lambda_2}^{mII} \varepsilon_{\lambda_1}^d) + (\varepsilon_{\lambda_1}^{mI} \varepsilon_{\lambda_2}^d - \varepsilon_{\lambda_2}^{mI} \varepsilon_{\lambda_1}^d) \times K_m] L} \quad (35)$$

$$C_{MB} = (\beta_1 A_{\lambda_1} + \beta_2 A_{\lambda_2}) [1 + K_m(T)] [1 + 2K_d(T) \times (\beta_1 A_{\lambda_1} + \beta_2 A_{\lambda_2}) (1 + K_m(T))] \quad (36)$$

$$E_T = \frac{1}{N \times M} \sum \{C_{MB} - [\alpha_1 A_{\lambda_1} + \alpha_2 A_{\lambda_2} + \alpha_3 A_{\lambda_3}]\}^2 + \frac{1}{N \times M} \sum \{C_{MB} - (\beta_1 A_{\lambda_1} + \beta_2 A_{\lambda_2}) [1 + K_m(T)] [1 + 2K_d(T) (\beta_1 A_{\lambda_1} + \beta_2 A_{\lambda_2}) (1 + K_m(T))]\}^2 \quad (37)$$

All these equations, based on the mass balance $C_{MB} = C_m + 2C_d = (C_{mI} + C_{mII}) + 2C_d$, revert back to the former ones if $K_m = 0$ ($C_{mI} = 0$). Thus, by maintaining the values of the thermodynamic parameters

previously evaluated (ΔS_d , ΔH_d , ΔS_m and ΔH_m ; Table 2), the minimization of the E_T error [eq. (37)] allows the values of ε_{664}^{mII} , ε_{610}^{mII} , ε_{664}^{mI} , ε_{610}^{mI} , ε_{664}^d , ε_{610}^d to be obtained in a first step. Subsequently, the molar attenuation coefficients in the whole wavelength range can be evaluated with the same routine that permitted to build the spectra in Figure 4. The results obtained are shown in Figure SI-1 of the Supporting Information. As displayed in the upper plot of the figure, the dimeric constant deviations and the error values are very low for wavelengths up to ~ 670 nm, a point from which the errors are clearly magnified. This may be the reason of the apparent discrepancy of the dimer spectrum at $\lambda > 670$ nm between Figures 4 and SI-1. As commented before, the sharp absorbance decrease to the right of the maximum (Fig. 1) provokes an appreciable variation in the absorbance by even the slightest deviation in the wavelength (the wavelength accuracy of the Uv-Vis spectrometer used in this work is ± 0.3 nm). In order to circumvent this issue, the minimization procedure was improved by letting the absorbance at a given wavelength oscillate within the curve formed by the absorbances at λ , $\lambda - 0.3$ and $\lambda + 0.3$ nm. The deviation ($\Delta\lambda$) for each triad of wavelength, MB concentration and temperature was treated as a fitting variable. Figure 8 displays the results. The upper plot shows the wavelength deviations averaged for all MB concentrations and temperatures at each wavelength. With this small correction, it was possible to reach extremely low values both for the E_T error and for the K_d deviations (middle plot of Figure 8). Finally, the lower plot of Figure 8 shows the refined spectra for the dimer, *mesomer I* and *mesomer II*. With the wavelength correction, the zone of the dimer spectrum at $\lambda > 670$ nm has now regained the appearance of the spectrum shown in Figure 4. The shapes of the spectra for both mesomers are also similar to the shapes of those previously obtained at the lowest concentration (Fig. 7A), which confirms the validity of the method. Since the spectra in Figure 8 were obtained with all the MB concentration and temperature data, they must be considered more refined than those of Figure 7A. The excellent goodness of fit can be also visualized in the remarkable coincidence between the experimental and

calculated absorbance curves displayed in Figure SI-2 of the Supporting Information. The molar attenuation coefficients at the maxima of each spectrum of Figure 8, as well as the values of the thermodynamic parameters are indicated in Table 2. It is noteworthy the presence of two maxima in the *mesomer I* spectrum, at 650 and 681 nm, placed at each side of the maximum for the *mesomer II* spectrum (664 nm). This peculiar characteristic of the *mesomer I* spectrum, which may be the reflection of two different chromophores in this resonance form, is responsible for the change of habit with temperature of the monomer spectra in different wavelength ranges (*i.e.* Figures 7D and 7E or Figure 5B). In conclusion, the methodology presented in this work together with the resonance virtual equilibrium hypothesis permit the visible light spectroscopic characterization of aqueous MB solutions at low concentration to be established with a high accuracy.

6. CONCLUSIONS

After a close examination to the most relevant literature on Methylene Blue self-aggregation in water, a novel procedure based on the Beer-Lambert equation has allowed precise visible-light absorption spectra and thermodynamic equilibrium parameters ($\Delta S_d = -43.3 \text{ J mol}^{-1} \text{ K}^{-1}$, $\Delta H_d = -34.4 \text{ kJ mol}^{-1}$) to be obtained for the monomeric and dimeric forms of this cationic dye in water. A thermo-solvatochromic effect detected in the monomer spectra is believed to be the main source of inaccuracy in all previous works. The existence of a colorless form of the monomer, proposed by Lewis *et al.* [12] to explain the solvatochromic effect, is refuted in this work. In its place, a new hypothesis has been raised; the resonance virtual equilibrium hypothesis. According to this hypothesis the temperature-dependent absorption behavior of the monomer is provoked by the change in its electron charge distribution with the variation in the temperature-dependent dielectric constant of water. The charge distribution stands between those of the virtual resonance forms, being the absorption spectrum of the monomer a

composition of the theoretical spectra for the virtual mesomers, whose proportion is established by a temperature-dependent equilibrium constant. Thanks to the application of this hypothesis, the virtual equilibrium parameters ($\Delta S_m = 24.0 \text{ J mol}^{-1} \text{ K}^{-1}$, $\Delta H_m = 8.1 \text{ kJ mol}^{-1}$) and molar attenuation coefficients for the mesomers were evaluated with remarkable accuracy.

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Captions to Figures

Figure 1. Absorption spectrum for an aqueous MB solution. Inset: Variation of A_{664}/A_{610} ratio for the different MB concentrations and temperatures analyzed in this work

Figure 2. Dimeric equilibrium constants at different temperatures obtained by minimization of the E_T error [eq. (21)] for $\lambda_1=664$ nm and $\lambda_2=610$ nm

Figure 3. Monomer fraction at different conditions evaluated by equation (22) for $\lambda_1=664$ nm and $\lambda_2=610$ nm

Figure 4. Upper plot: dimeric equilibrium constants at different temperatures and minimization error [eq. (21)] obtained in the 500–695 nm wavelength range. Lower plot: absorption spectra for the dimer and monomer at different temperatures obtained by minimization of the E_T error [eq. (21)]

Figure 5. Symbols: evolution with the temperature at different wavelengths of the observed molar attenuation coefficients in the absence of dimer for (A) data by Lewis *et al.* [12] ($C_{MB}=6.3\times 10^{-7}$ mol L⁻¹) and (B) data obtained in this work ($C_{MB}=1.1\times 10^{-6}$ mol L⁻¹). Solid lines: molar attenuation coefficients predicted by the hypothesis of resonance virtual equilibrium [equation (31)] for $\Delta S_m = 24.0$ J mol⁻¹ K⁻¹ and $\Delta H_m = 8.1$ kJ mol⁻¹

Figure 6. Resonance forms of cationic Methylene Blue with values and directions of the theoretical (μ_G and μ_E) and experimental ($\mu_{G,EXP}$ and $\mu_{E,EXP}$) moment dipoles for the ground (G) and excited singlet (E) states

Figure 7. A- Molar attenuation coefficients of *mesomers I* and *II* determined by fitting equation (31) to the experimental molar attenuation coefficients obtained at $C_{MB}=1.1\times 10^{-6}$ mol L⁻¹. B- Experimental molar attenuation coefficients at $C_{MB}=1.1\times 10^{-6}$ mol L⁻¹ (symbols) and those evaluated by equation (31)

according to the hypothesis of resonance virtual equilibrium (lines). C to E- Augmented areas of Figure 7B

Figure 8. Upper plot: wavelength deviations employed to minimize the E_T error [eq. (37)]. Middle plot: dimeric equilibrium constants at different temperatures and minimization error [eq. (37)] obtained in the 500–695 nm wavelength range. Lower plot: absorption spectra for the dimer, *mesomer I* and *mesomer II* obtained by minimization of the E_T error [eq. (37)]

Tables

Table 1. Literature values of optical and thermodynamic parameters of MB in water at low concentration

Ref.	λ_1 nm	λ_2 nm	$\epsilon_{\lambda_1}^m$ ($\times 10^{-4}$) L mol ⁻¹ cm ⁻¹	$\epsilon_{\lambda_1}^d$	$\epsilon_{\lambda_2}^m$	$\epsilon_{\lambda_2}^d$	T °C	$K_d \times 10^{-3}$ L mol ⁻¹	ΔH_d kJ mol ⁻¹	ΔS_d J mol ⁻¹ K ⁻¹
[11]	656.5	600.0	4.00	2.04	2.07	6.28	27	3.57	-	-
[17]	-	-	-	-	-	-	25	2.59	-	-
[12] ^a	660.0	-	8.69 ^b	-	-	-	9	-	-	-
			8.44 ^b	-	-	-	23			
			8.15 ^b	-	-	-	40			
			7.86 ^b	-	-	-	60			
[1]	667.0	-	5.83	-	2.90	-	30	-	-	-
[13] ^a	662.0	-	8.50	-	3.06	-	27	2.50	-	-
[6] ^a	664.0	605.0	9.56	2.44	3.81	13.06	25	5.88±0.50	-	-
[14]	668.0	-	9.00	-	-	-	20	4.65±0.28	-22.2±6.3	+4.2±2.5
							30	3.28±0.20		
							40	2.51±0.15		
							50	2.03±0.13		
[15]	660.0	610.0	7.18	1.54 ^c	3.88	9.06	20	3.97±0.47 ^d 4.67±0.52 ^e	-83.2±10.5	-253.8±16.7
[9] ^a	664.0	610.0	6.61	8.94	3.02	8.18	10	33.11 ^f	-46.8 ^g	-80.0 ^h
							20	15.49 ^f		
							40	3.98 ^f		
							60	1.38 ^f		
This work ⁱ	664.0	610.0	7.98	3.53	3.28	10.47	9	12.68±0.16 ^j	-34.4 ^k	-43.3 ^k
			7.66		3.34		23	6.26±0.09 ^j		
			7.33		3.33		40	2.91±0.15 ^j		
			6.98		3.30		60	1.31±0.11 ^j		

^a Molar attenuation coefficients data extracted from figures; ^b $C_{MB}=6.3 \times 10^{-7}$ M; ^c standard deviation = $\pm 3.5 \times 10^4$ (very much higher than for the other three coefficients); ^d evaluated at $\lambda=610$ nm; ^e evaluated at $\lambda=660$ nm; ^f $C_{MB}=3.0 \times 10^{-5}$ M; ^g average value (from -54.6 to -38.9); ^h average value (from -104.0 to -56.0); ⁱ $E_T = 1.6 \times 10^{-14}$ [eq. (21)]; ^j eq. (19); ^k eq. (20)

Table 2. Optical and thermodynamic parameters of MB in water at low concentration

Optical parameters			Thermodynamics	
Species	λ_{max} nm	$\epsilon_{\lambda_{max}} \times 10^{-4}$ L mol ⁻¹ cm ⁻¹	Monomer / Dimer	
Dimer	606	10.45	ΔS_d (J mol ⁻¹ K ⁻¹)	-43.3
Monomer <i>mesomer II</i>	664	10.78	ΔH_d (kJ mol ⁻¹)	-34.4
Monomer <i>mesomer I</i>	650 681	4.40 4.17	Mesomer II / Mesomer I	
			ΔS_m (J mol ⁻¹ K ⁻¹)	24.0
			ΔH_m (kJ mol ⁻¹)	8.1

Figures

Figure 1

Figure 2

Figure 3

Figure 4

Figure 5

Figure 6

Figure 7

Figure 8