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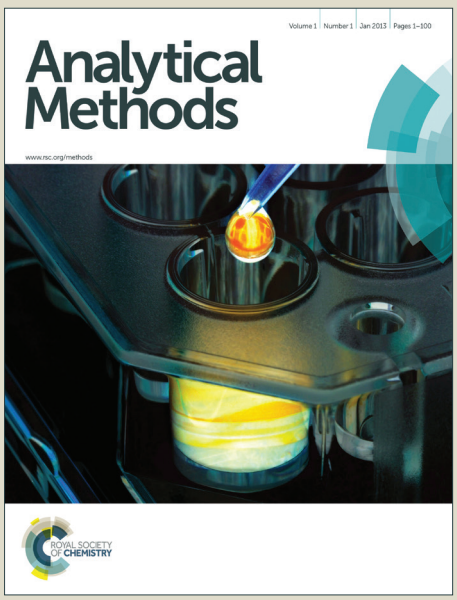

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Solution scanometry, a new method for determination of acidity constants of indicators

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Abstract A new, simple, fast and inexpensive method as solution scanometry was applied for the determination of the acidity constant, pK_a, of indicators, for the first time. Three azo indicators including Methyl orange (MO), Methyl yellow (MY) and Methyl red (MR) were selected as proper templates of indicators for this purpose. The method is based on scanning of the cells containing the indicator solution with a scanner and then the color of each cell is analyzed with software written in visual basic (VB 6) media to red, green and blue values. The cells were built by creating holes in the Plexiglas® sheet. Also the acidity constants of the cited three azo indicators were determined spectrophotometrically. The pK_a of the indicators was evaluated using spectrophotometric data by the Hypspec program. The corresponding distribution diagrams were depicted through Hyss2009 program. The comparison between the current and traditional Uv-Vis spectrophotometry method was studied and the results revealed similar trends in both methods. The results obtained from two methods have a good agreement with literature.

Keywords Solution scanometry. Acidity constants. Plexiglas® cell. Hypspec program. Azo indicators

1. Introduction

Dissociation constants (i.e. pK_a values) can be a key parameter for understanding and quantifying chemical phenomena such as reaction rates, biological activity, uptake and transport,[1, 2] developing pre-clinical and clinical formulation,[3] environmental fate and are helpful in screening salts. Also pK_a is one of the important physicochemical properties of drugs in the analysis of them, in the interpretation of their mechanisms of action, to understand their site of absorption, distribution to various organs and excretion.[4-6] The acidity constants of organic reagents play a very fundamental role in many analytical procedures

such as acid-base titration, solvent extraction, complex formation[7-12] and in developing other analytical methods, like HPLC.[5]

Several methods have been introduced for determining acidity constants. These methods include spectrophotometric determination,[13] potentiometric titration,[14] calorimetric methods,[15] proton magnetic resonance (1H NMR) spectroscopy,[16] (Fourier transform-infrared) FT-IR spectrometry,[17] fluorescence spectrophotometry,[18] conductometry,[19] capillary electrophoresis[20] and chromatography.[21] Spectrophotometric method is most useful and widely used. The most widely used programs and algorithms for determining acidity constants from absorbance data are LETAGROP-SPEFO [22], SQUAD [23], SPECFIT [24], STAR [25], SPECA [26], pHab [27, 28] and DATAN.[10] In this work, solution scanometric method was introduced for the evaluation of pK_a of indicators, for the first time and the results were compared with the spectrophotometry results obtained by HypSpec program, a new version of the pHab program [27, 28] and from literature [29-34]. HypSpec is a new program for calculation of pK_a of ligands [35, 36] and stability constants of complexes in aqueous and nonaqueous solution, using spectrophotometric data.[37-39]

The solution scanometric technique was introduced recently by Abbaspour et al.[40] The method is based on scanning of Plexiglas® cells containing colored solutions. The Plexiglas® cells were scanned and finally the RGB (red, green and blue) color model was used in color monitor.[41-44] This method was developed by shokrollahi et al. to CPE-Scanometry.[45]

Advantages of solution scanometry include simplicity (handheld scanner and PC), high scanning speed, inexpensive, portable systems and easy immobilizing of reactants, no need for finding the λ_{max}, intensing archive of experiences, short response time, limiting the interferences, capability of various simultaneous tests and using non-transparent solution and investigation of the reflective properties of the surface. However, there are disadvantages such as the lack of uniformity in the membrane, that causes serious effects on the relative standard deviation percent and precision of analysis.

The basic compounds of the azo indicators (Fig.1) are p-amino-azobenzene, or p-dimethylamino-azobenzene (dimethyl yellow), both of which are insoluble in water. Compounds soluble in water are obtained if polar groups such as the sulphonic acid (methyl orange) or carboxylic acid groups (methyl red) are introduced into the molecule. The classical azo indicators are red in acid and yellow in alkaline medium. The colour change occurring in acid medium is caused by formation of the indicator-cation. Because of their unshared electron-pairs, the nitrogen atoms of the azo group are capable of binding protons, thus causing the formation of a quinonoid benzene ring respectively of a resonance system. In acid

medium, the so-callede "Zwitter"-ion structure is formed; therefore the salt error of many azo indicators is negligible; consequently they are suitable for colorimetric determination of pH.[46, 47]

2. Experimental

2.1. Apparatus

The cells (with 1000 µL volume for each of them) were built by using a sheet of Plexiglas®. A Canoscan LiDE 200 flatbed scanner was used for scanning thePlexiglas®sheet. The resolution of the scanner was regulated at 300 dpi. A Biohit prolinepipettor 100-1000 µL was used for injecting samples into the cells. A Metrohm (Herisau, Switzerland) digital pH meter Model 827 with a combined glass electrode was used to measure pH values. Absorbance measurements were made by Biochrom (Biochrom, Cambridge, UK.), Uv-Vis spectrophotometer equipped with 1-cm quartz cells.

2.1.1. Preparation of cells array

In this method we used the cylindrical cells (Fig. 2). These cells were built by creating holes (id 1.5 cm) in the sheet of Plexiglas® (thickness 0.5 cm), using laser. In order to close the bottom of the holes and make the cells, this holed sheet was stuck to another sheet of Plexiglas® (thickness of 0.1 cm).
In our design, the cells were aligned to 3 columns and 7 rows, giving a total of 21 cells in the Plexiglas®sheet.

2.2. Materials

All chemicals used in this work were of analytical grade. In addition, double distilled water was used throughout. MO, MY and MR were_purchased from M/s Merck (E. Merck, Darmstadt, Germany). Laboratory stock solutions (2.0×10^{-3}, 5.0×10^{-3} and 2×10^{-3} mol L^{-1} for MO in water, MR in methanol and MY in ethanol respectively) were made by dissolving proper amounts of each indicator in 50 ml solvent. Analytical-grade of hydrochloric acid, sodium hydroxide, potassium nitrate and methanol used in this study were obtained from Merck Company.

2.3. Softwares

The VB based special software[48] was used to convert the recorded pictures of color of cells to RGB data. HypSpec, a new version of pHab program[49] and Hyss 2009, a new edition of the older version[50] were used to acquire and analysis data.

2.3.1 Principles of the red, green and blue (RGB) color system

The RGB color model is an additive color model in which red, green, and blue lights are added together in various ways to reproduce a broad array of colors. In computing, the color values are often stored as integer numbers in the range 0 to 255, the range that a single 8-bit byte can offer (by encoding 256 distinct values). In the RGB system, any color is represented in the form of (R, G, B), in which the (0, 0, 0) and (255, 255, 255) refer to black and white, respectively. Therefore, by increasing the intensity of colors, the color values are decreased. In this system16777216 colors can be made. Any color can be described by the following formula:

$$V = R + 256\,G + 256^2\,B$$

Where, R, G and B are red, green and blue values of the main color. For black and white, V is equal to 0 and 16777216, respectively. By using the following flowchart, R, G and B values of V for any color can be extracted:

$$R = V \bmod 256$$
$$G = ((V-R) \bmod (256^2)) / 256$$
$$B = (V - R - G*256) / (256^2)$$

‘‘Mod’’ is a numeric function which returns the remainder when dividing two numbers.

2.3.2 Hypspec program

HypSpec is a program in the Hyperquad suite and shares some features with other programs in the suite. HypSpec can be used to derive equilibrium constants from spectrophotometric data. The data may be in the form of one or more titration curves, or single data points (batch data). pH measurements may optionally be included in the experimental data. The program combines the functionality of Hyperquad2006, a new version of Hyperquad[51] and pHab[27, 28],

but with some simplifications. The basic algorithm in pHab has been reported in the literature [27, 28].

Some of the key developments in HypSpec program are the following. It has a new program structure (written in Visual Basic6 instead of FORTRAN module); data are "live" when performing a refinement and it has improved facilities for the use of batch and titration data, refinement output simplified, and special adaptations when data include pH etc. are some other features.

The use of the algorithm for computing equilibrium constants in HypSpec involves the following sequence:

1. Start with a set of known and estimated overall protonation or stability constants (β).

2. Compute the objective function (sum of squares, U) as in Eq. (1)

$$U = \sum_{i=1,np} W_i r_i^2 \qquad (1)$$

where W is a (diagonal) weight and r is a residual, equal to the difference between the observed and calculated values.

3. Change the unknown protonation or stability constants and repeat the calculations until no further reduction of U (i.e., the sigma in HypSpec output, has been minimized) can be obtained.

$$\sigma = (U/np - n)^{1/2} \qquad (2)$$

Where np-n, the number of data points minus the number of parameters is the expectation value of the objective function.

When systematic errors are present, the weighted sum of squares will be more than np-n. Good experimental practice seeks to reduce systematic errors to a level where they are small compared with random errors. HypSpec has a facility for calculating the weights based on estimates of the experimental errors.

4. At the end, providing the final calculated log β values with standard deviation can be accepted as the final results.

2.4. Procedure

In this section two procedures including solution scanometric and spectrophotometric method were employed to obtain acidity constants of the three azo indicators (MO, MY and MR). Each procedure was the same for the cited indicators except the solvent and concentration. Here, the procedure is described for MO, as an example.

2.4.1. Solution scanometric method

The stock solution of MO (2.0×10^{-3} mol L^{-1}) was prepared in water. The first step in this study involves preparing two solutions of HMO (acidic form) and MO^- (conjugate base). The first solution (A) was prepared by diluting a mixture of 1 ml of the standard MO solution and 0.1 mol L^{-1} HCl to 25 ml. The pH of this solution is about 1, so the MO is present entirely as HMO. The second solution (B) was prepared by diluting a mixture of 1 ml of the standard MO solution and 0.1 mol L^{-1} NaOH to 25 ml. The pH of this solution is about 13, so the MO is present entirely as MO^-.

The second step involves verifying the linear relation between the effective intensity and concentration of HMO and MO^-, and determining the slopes of linear ranges for G and B parameters. In order to obtain the linear range for acidic form (HMO), several dilute solutions with concentration between $1.6 \times 10^{-3} - 8.0 \times 10^{-2}$ mol L^{-1} in present 0.1 mol L^{-1} KNO_3 as the supporting electrolyte, were prepared from (A) solution. For the basic form (MO^-), it was carried out similar but from (B) solution. The effective intensity-concentration calibration curves are shown in Fig. 3.

The third step in the experiment is to determine the relative amounts of HMO and MO^- present in the solution as a function of pH. A series of dye solutions with pHs 3.23, 3.43, 3.57 and 4.01 were prepared by adding varying amounts of HCl and NaOH. Following that, effective intensity of G, B parameters of the solutions was measured. 500 µL of these solutions was injected into each cell. After shaking, the color cells were scanned with the scanner; the image was transferred into the computer and any color changes in each cell were analyzed, using a program written in VB 6 media. In this program, the color of each cell is analyzed based on the RGB system into R, G and B values. It should be noted that in the color analyzing programs, a specific area was selected for analyzing and the number of pixels that could be indicated by this area was about 10000– 300000. This program can average these pixels. Therefore, the noise can be dramatically decreased. The effective intensity for any color values was calculated as follows:

$$A_r = \log (R_s/R_b)$$

$$A_g = \log (G_s/G_b)$$

$$A_b = \log (B_s/B_b)$$

Where, A_r, A_g and A_b are the effective intensity for red, green and blue respectively. R_s, G_s, B_s and R_b, G_b and B_b are the red, green and blue color values of a sample and a blank, respectively.

Also, for the other two indicators, the procedures were carried out in a similar way except for MY, was performed in 50:50, water: ethanol solvent. The results are shown in Table 1.

2.4.2. Spectrophotometric method

The acidity constant of MO was easily obtained using 2.4×10^{-5} mol L^{-1} solutions of it, in pH range 1.0-13.0 at 25 ℃. After each pH adjustment by hydrochloric acid and sodium hydroxide, the proper amount of solution was transferred into the quartz cell and the absorption spectra were recorded. In all the experiments, the ionic strength of the solutions was kept constant at 0.1 mol L^{-1} using potassium nitrate as the supporting electrolyte. The obtained absorbance-pH data was used for calculation of protonation constant by HypSpec program. It was done for the MY and MR in a similar way in corresponding solvents.

3. Results and discussion

3.1. Determination of the acidity constant of indicators by solution scanometric method

Acid-Base indicators (also known as pH indicators) are substances which change color with pH. They are usually weak acids or bases, which, when dissolved in water, dissociate slightly and form ions. Consider an indicator which is a weak acid, with the formula HIn. At equilibrium, the following equilibrium equation is established with its conjugate base:

$$HIn + H_2O \rightleftarrows H_3O^+ + In^- \qquad (3)$$

Here HIn stands for the acid form and In^- for the conjugate base of the indicator. The acid dissociation constant, K_a, is defined as:

$$K_a = \frac{[H^+][In^-]}{[HIn]} \qquad (4)$$

Converting Equation 4 into the form of the Henderson-Hasselbach equation:

$$pK_a = pH - \log\frac{[In^-]}{[HIn]} \qquad (5)$$

The acid dissociation constant may be calculated from measurements of the ratio $[In^-]/[HIn]$ at known pH values.

The new pK_a determination method described here is simple, fast and inexpensive. The ratio $[In^-]/[HIn]$ is calculated from the following equations: When pH is less than pK_a , the indicator is mainly in the acidic form, and the species HIn is responsible for the effective intensity recording, A_{HIn}. When pH is greater than pK_a, the indicator is mainly in the basic form, and the species In^- is responsible for the effective intensity recording, A_{In^-}.

$$A^g = A^g_{HIn} + A^g_{In^-} \quad (6)$$

$$A^b = A^b_{HIn} + A^b_{In^-} \quad (7)$$

Where A^b and A^g are the effective intensity of B and G, respectively.

$$A^g = S^g_{HIn} C_{HIn} + S^g_{In^-} C_{In^-} \quad (8)$$

$$A^b = S^b_{HIn} C_{HIn} + S^b_{In^-} C_{In^-} \quad (9)$$

S^b and S^g are slope of blue and green color *vs.* conc. calibration graphs, respectively, and C_{HIn} and C_{In^-} are concentration of the acidic (HIn) and basic (In^-) forms of indicator.

By solving both Equations (8) and (9) simultaneously, one can obtain concentrations of the HIn and In^-.

$$C_{HIn} - \frac{S^b_{In^-} \times A^g - S^g_{In^-} \times A^b}{S^g_{HIn} \times S^b_{In^-} - S^g_{In^-} \times S^b_{HIn}} \quad (10)$$

$$C_{In^-} = \frac{S^g_{HIn} \times A^b - S^b_{HIn} \times A^g}{S^g_{HIn} \times S^b_{In^-} \; S^g_{In^-} \times S^b_{HIn}} \quad (11)$$

From the amounts of HIn and In^- present as a function of pH the pK_a value for indicators can be calculated using Equation (5).

3. 2. Determination of acidity constants of azo indicators by solution_scanometric method

The objective of this study is to determine the acid dissociation constant for azo indicators, using solution scanometric method and the comparison of results with those of the spectrophotometric method. MO was selected for detailed investigation. In aqueous solution,

MO is a zwitterion and has a resonance structure somewhere between the two extreme forms (Fig. 1). In acidic solution, it has a bright red color (HMO form) which changes to yellow in its basic form (MO^-). Based on the above equations (3-11) and description, the acidity constant of MO^- can be calculated by solution scanometric method.

Calibration graphs were constructed to determine the concentration of the species of interest, particularly HMO and MO^-. As it is seen from Fig. 3, the equation of the regression line for acidic and basic form (for G parameter) were $A^g = 5977.3\ C_{HMO} + 0.0129$ and $A^g = 384.4\ C_{MO^-} + 0.0288$ and for B parameter were $A^b = 4818.4\ C_{HMO} + 0.0105$ and $A^b = 5896.3\ C_{MO^-} + 0.03$, respectively. The concentration of species is in mol L^{-1}.

$$A^g = 5977.3\ C_{HMO} + 384.4 C_{MO-} \quad (12)$$

$$A^b = 4818.4\ C_{HMO} + 5896.3\ C_{MO-} \quad (13)$$

From G and B effective intensities at each pH of pHs in Table 2, and Equations (12) and (13), the ratio concentration of HMO to MO^- in solution can be calculated. The value of pK_a for MO was evaluated from the ratio concentration of HMO to MO^- as a function of pH, using Equation (5). The results are shown in Table 2. Two other indicators were studied similarly and the results are given in Tabels 3 and 4.

3. 3. Determination of acidity constant of azo indicators by spectrophotometric method

Spectrophotometric methods are, in general, powerful, highly sensitive and suitable for studying chemical equilibria solution. These methods are attractive for pK_a determination in very dilute aqueous solutions. Thus, the spectrophotometric is a reliable method for confirming the results obtained from the new methods. Therefore, the new results from solution scanometric method were compared with the spectrophotometric results.

Subsequently, UV-Vis spectrophotometry was used to obtain spectral data (Fig. 4) and then commercially available HypSpec software was used, which allowed us to perform spectral subtraction followed by spectral analysis to determine the acidity constants. Acidity constants and molar absorbance spectra (Fig. 5) of three azo indicators were evaluated by HypSpec program using absorbance- pH data. Also the corresponding distribution diagrams were depicted by Hyss2009 (Fig. 6). The results are shown in Tables 2, 3 and 4.

According to these Tables, the significant difference between the purposed method and spectrophotometric study was tested by F-test at p=0.05. The test ascertained that there was

no significant difference among the methods, and the results obtained from solution scanometric method have a good agreement with the spectrophotometric results and the results reported in the literature.[29-34]

4. Conclusion

Solution scanometric method as an easy, safe, rapid, portable, and an inexpensive method was introduced for determining the acidity constant, pK_a, of indicators, for the first time. This method was developed as an alternative to visible spectrophotometry. The acidity constants of three azo indicators including MO, MY and MR were obtained by the proposed method for the first time. The results are very satisfactory in comparison with spectrophotometric results and previous reports. [29-34]

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Figure captions

Fig. 1. Structure of three azo indicators: MO (X=SO_3^-, Y=H), MY (X=H, Y=H) and MR (X=H, Y=CO_2^-), acidic form (a), basic form (b)

Fig. 2. Schematic of cell array on the Plexiglas® sheet and solutions of MR as a sample of three azo indicators at various pHs

Fig. 3. Calibration graph for MO, acidic form (a), basic form (b)

Fig. 4. Absorption spectra of three azo indicators: MO (a), MY (b) and MR (c) at different pH values

Fig. 5 Molar absorbance spectra of three azo indicators: MO (a), MY (b) and MR (c)

Fig. 6 The corresponding distribution curve of three azo indicators: MO (a), MY (b) and MR (c)

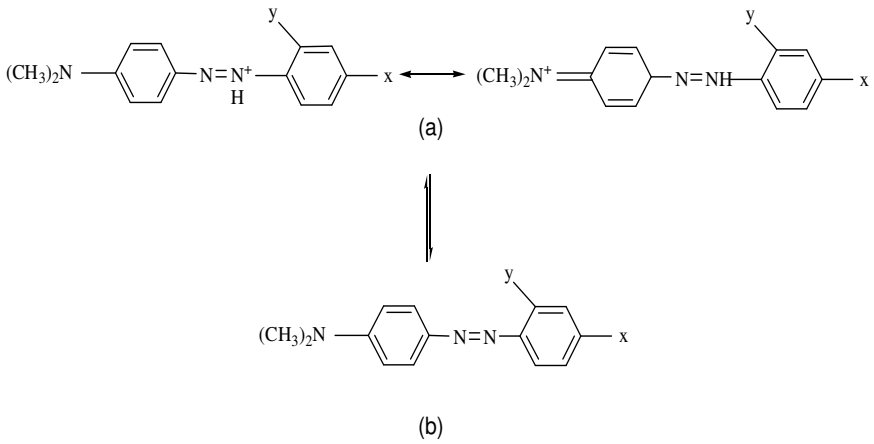


Fig. 1.

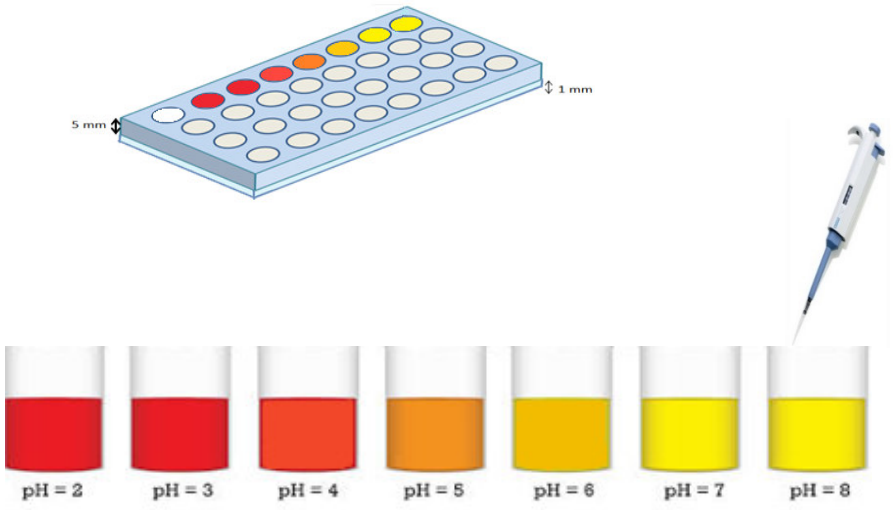
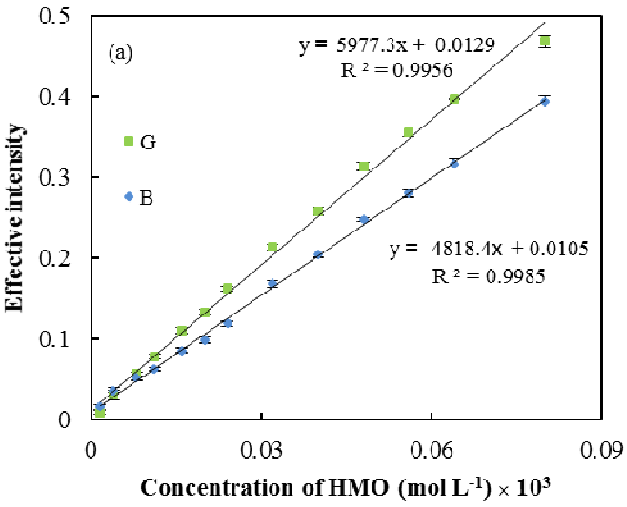


Fig. 2.



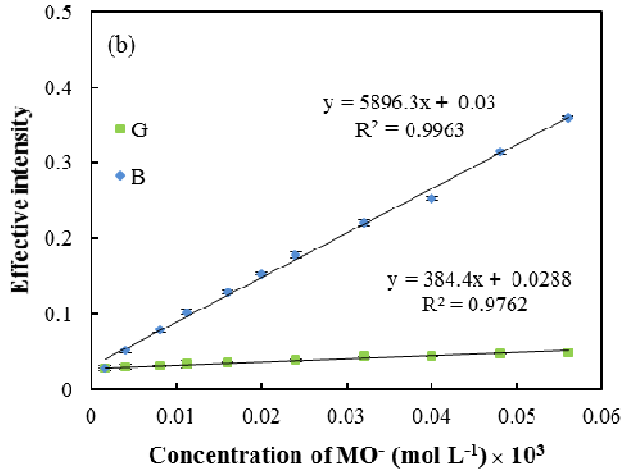


Fig. 3.

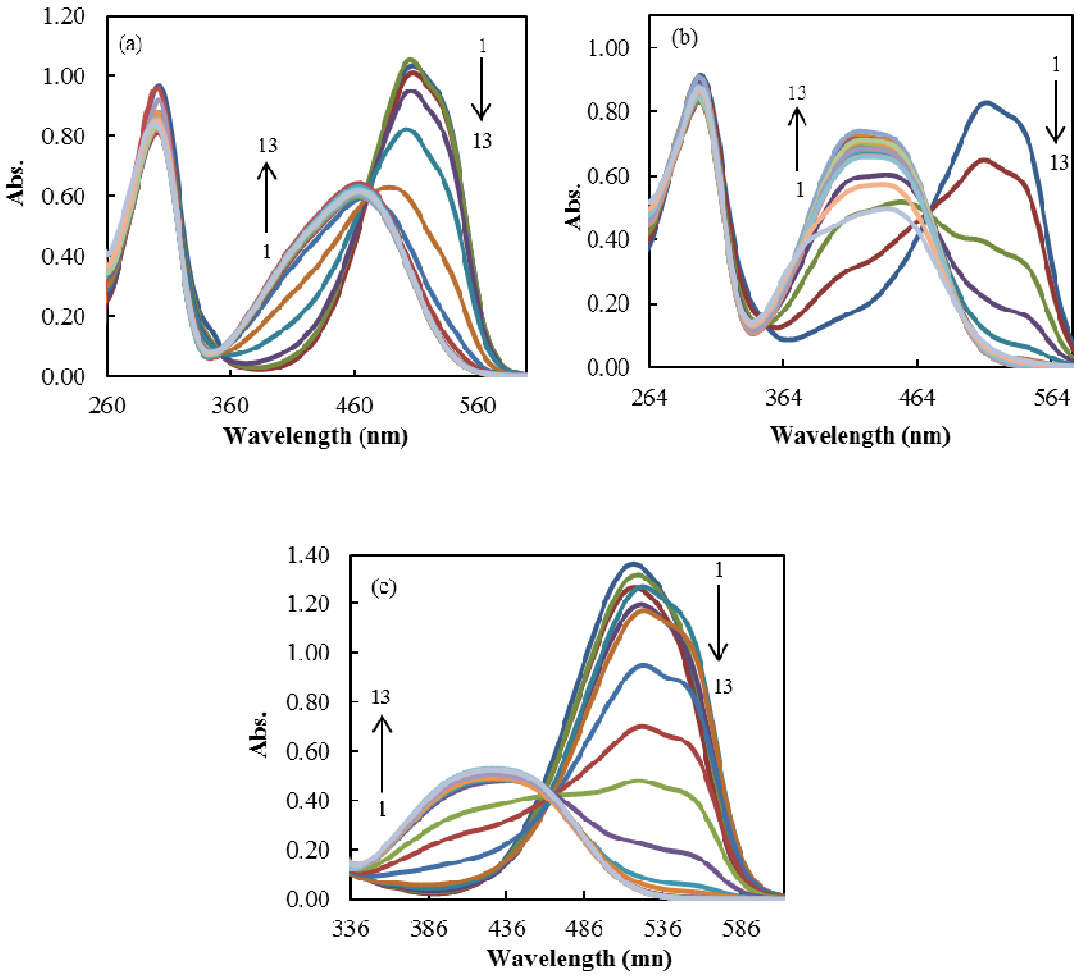


Fig.4.

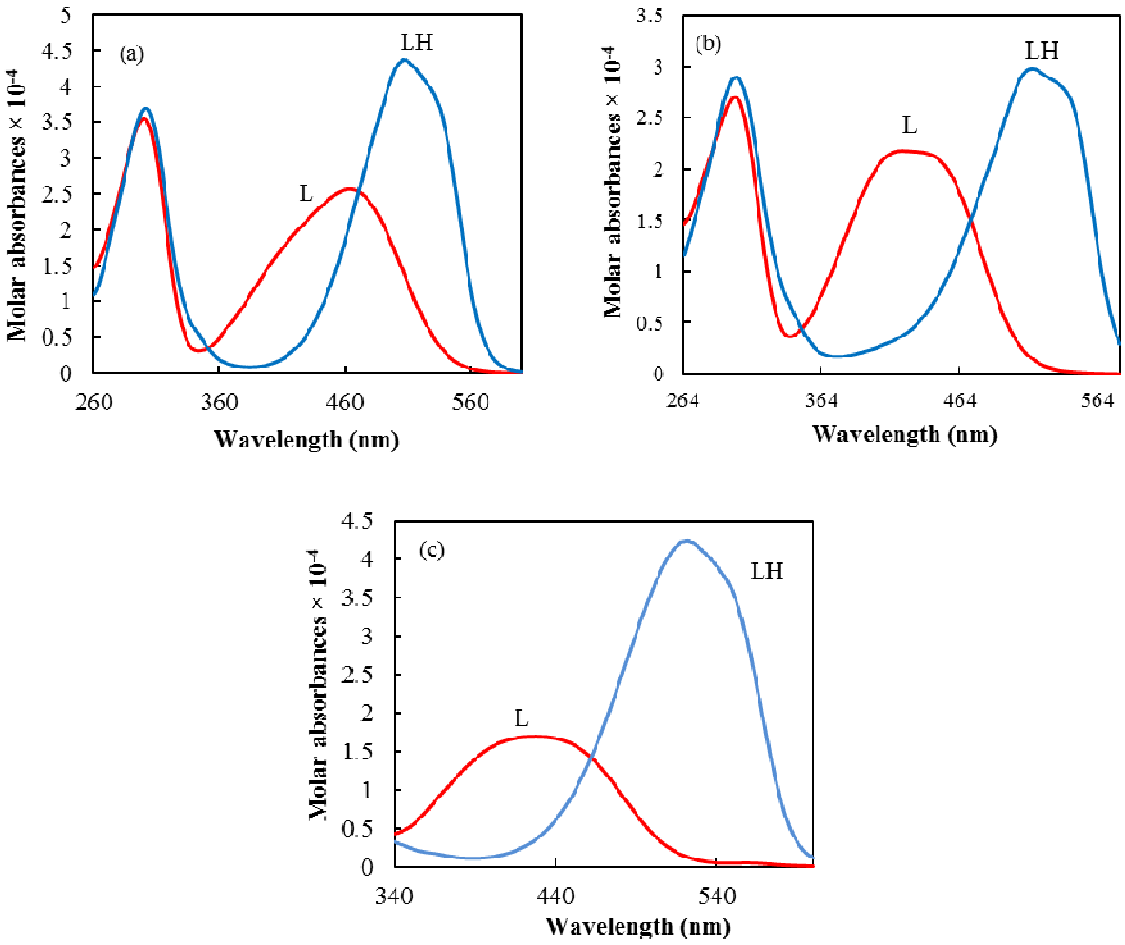


Fig.5.

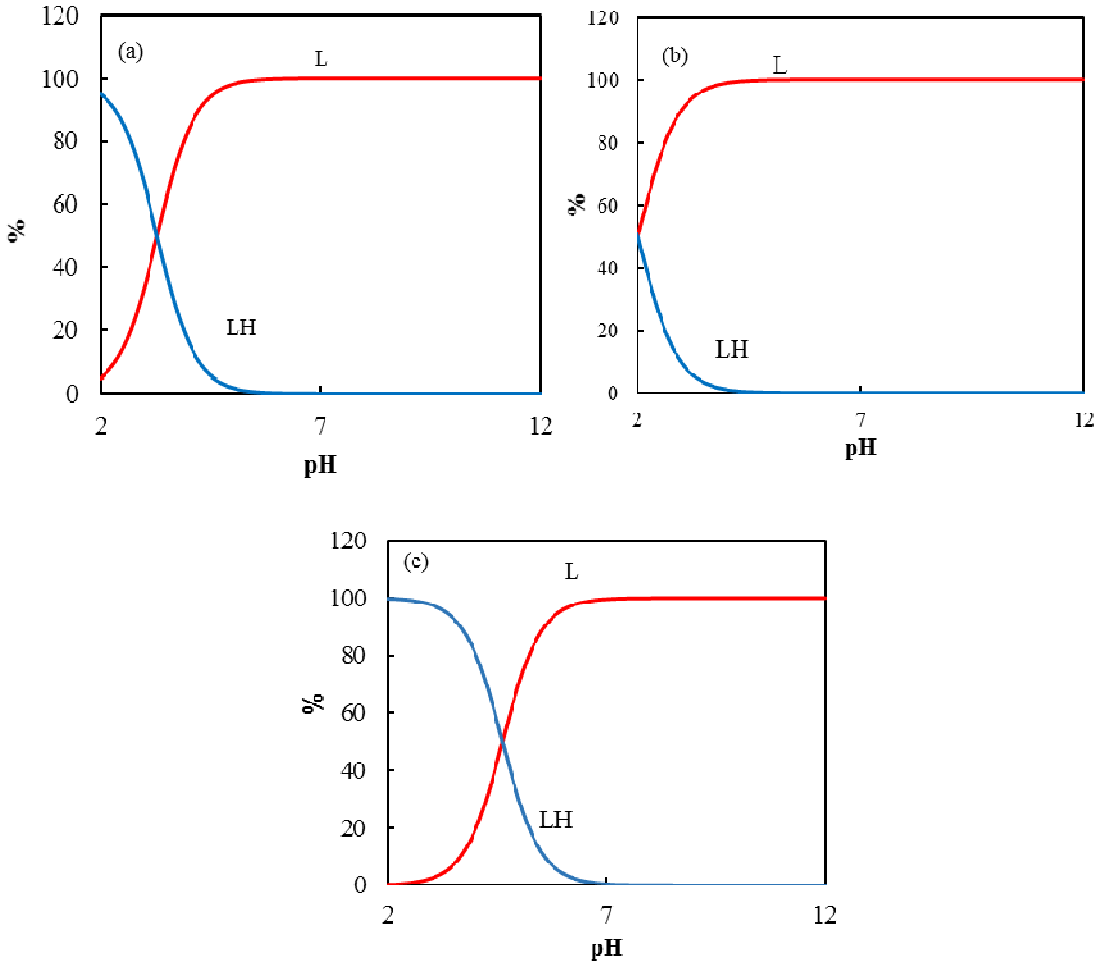


Fig.6.

Table 1 The calibration equations for three azo indicators obtained by solution scanometric method

Indicartors	Acidic form		Basic form	
	G parameter	B parameter	G parameter	B parameter
Methyl orange	Y=5977.3C+0.0129	Y=4818.4C+0.0105	Y=384.4C+0.0288	Y=5896.3C+0.03
Methyl yellow	Y=4921.2C+0.0012	Y=2109.4C+0.0262	Y=156.1C+0.0073	Y=4026.3C+0.0153
Methyl red	Y=8217C+0.0138	Y=3230.2C+0.0134	Y=0	Y=3961.2C-0.0039

Table 2 Acidity constant value of MO calculated by solution scanometric and spectrophotometric methods

Ref.	Method	pH(s)	pK_a	F	Decision $F_{0.05}(3,2)=19.16$
This work	Solution scanometry	3.23 3.43 3.57 4.01	3.04 3.07 3.07 3.10	6.00	Not differ significantly
	Spectrophotometry	1-13	3.24		
29	Spectrophotometry	-	3.34	-	
30	Spectrophotometry	-	3.46	-	

Table 3 Acidity constant value of MY calculated by solution scanometric and spectrophotometric methods

Ref.	Method	pH(s)	pK_a	F	Decision $F_{0.05}(2,3)=9.55$
This work	Solution scanometry	1.84 1.99 2.21 2.43	1.85 1.86 1.86 1.86	4.00	Not differ significantly
	Spectrophotometry	1-13	2.01		
31	Spectrophotometry	-	1.89	-	

Table 4 Acidity constant value of MR calculated by solution scanometric and spectrophotometric methods

Ref.	Method	pH(s)	pK_a	F	Decision $F_{0.05}(2,3)=9.55$
This work	Solution scanometry	5.0 5.0 5.47 5.93	4.72 4.73 4.73 4.74	1.50	Not differ significantly
	Spectrophotometry	1-13	4.62		
32		-	4.8	-	
33	Spectrophotometry	5.82 5.50 5.09 4.85	5.00 5.01 5.01 5.06	-	
34	Spectrophotometry	5.80 5.22 5.48	4.91 5.02 5.05	-	