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Color is a quality of substance and systems under study that can be reproducibly codified by digital systems using controlled conditions. Thus, the intensity of color is an appropriate parameter for the performing of analyses based on the quantitative monitoring of color changes. The objective of this work was to develop and evaluate a quantitative parameter which permits to carried out the color intensity description of analytical samples for its application in the digital image quantitative analysis (DIQA) and to show its application in the construction of McFarland scale for the determination of colony forming units (CFU) in the microbiology analysis. For that, a surface color index (I_{sc}) was defined from RGB color space. I_{sc} was used as a descriptor of color changes and it was evaluated

Keywords

McFarland Scale
RGB model
Digital image
Color

using different solutions of dyes with concentration between 2.0 and 100 mg/L. Results show that is possible to do linear calibration curves for the different test dyes, independently of color used. Detection and quantification limits were 9.9 and 3.2, respectively. In addition, mean relative error percentage, mean standard deviation and global coefficient of variation were 9.1 %, 3.16 mg/L and 13.7 %, in that order. It is concluded that surface color index is an adequate parameter for the monitoring of color from digital images in RGB format. In addition, it was verified that DIQA can be used in the determination of CFU, with an adequate correlation with the implementation of McFarland scale by ultraviolet-visible spectroscopy ($R^2 = 0,9986$).

Determinación de la concentración celular de microorganismos mediante análisis cuantitativo de imágenes digitales (ACID)

Resumen

El color es una cualidad de las sustancias que puede ser codificado de forma reproducible mediante sistemas digitales bajo condiciones controladas. Así, la intensidad del color es un parámetro apropiado para la realización de análisis basados en el monitoreo cuantitativo de los cambios del color. El objetivo del presente trabajo fue desarrollar y evaluar un parámetro cuantitativo que permita realizar la descripción de la intensidad del color de muestras analíticas para su aplicación en el análisis cuantitativo de imágenes digitales (ACID) e ilustrar su aplicación en la construcción de la escala McFarland para la determinación de las unidades formadoras de colonia (UFC) en el análisis microbiológico. Para ello, se definió un índice de color superficial (I_{cs}) a partir del espacio de color RGB. El I_{cs} se usó como descriptor de los cambios de color y se evaluó empleando soluciones de diferentes colorantes con concentraciones entre 2.0 y 100 mg/L. Los resultados muestran que es posible la construcción de curvas de calibración lineales para los diferentes colorantes de prueba, independientemente del color empleado. Los límites de detección y cuantificación fueron 9.9 y 33.2, respectivamente. Además, el error relativo porcentual promedio, la desviación estándar media y los coeficientes de variación globales fueron 9.1 %, 3.16 mg/L y 13.7 %, respectivamente. Se concluyó que el índice de color superficial es un parámetro adecuado para el monitoreo del color a partir de imágenes digitales en formato RGB. Además, se verificó que el ACID puede ser usado en la determinación de las UFC, con una adecuada correlación con la implementación de la escala McFarland mediante espectroscopia de ultravioleta-visible ($R^2 = 0,9986$).

Palabras claves

Escala McFarland
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Determination of microorganism cellular concentration by digital image quantitative analysis

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Resumen

Color is a quality of substance and systems under study that can be reproducibly codified by digital systems using controlled conditions. Thus, the intensity of color is an appropriate parameter for the performing of analyses based on the quantitative monitoring of color changes. The objective of this work was to develop and evaluate a quantitative parameter which permits to carried out the color intensity description of analytical samples for its application in the digital image quantitative analysis (DIQA) and to show its application in the construction of McFarland scale for the determination of colony forming units (CFU) in the microbiology analysis. For that, a surface color index (I_{sc}) was defined from RGB color space. I_{sc} was used as a descriptor of color changes and it was evaluated using different solutions of dyes with concentration between 2.0 and 100 mg/L. Results show that is possible to do linear calibration curves for the different test dyes, independently of color used. Detection and quantification limits were 9.9 and 3.2, respectively. In addition, mean relative error percentage, mean standard deviation and global coefficient of variation were 9.1 %, 3.16 mg/L and 13.7 %, in that order. It is concluded that surface color index is an adequate parameter for the monitoring of color from digital images in RGB format. In addition, it was verified that DIQA can be used in the determination of CFU, with an adequate correlation with the implementation of McFarland scale by ultraviolet-visible spectroscopy ($R^2 = 0,9986$).

Palabras Claves

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1. Introduction

The digital image quantitative analysis (DIQA) refers to the set of techniques for obtaining data from images of the studio system. At present, there are many techniques that allow the construction of images by analyzing different regions of the electromagnetic spectrum (visible, infrared,

microwave, etc.). Thus, for example, in combination with scanning electron microscopy, transmission electron microscopy and atomic force microscopy, the DIQA is used to evaluate the roughness, porosity and topological changes, the size and size distribution of pores in various surfaces [1]. However, in combination with microscopic techniques, the DIQA only allows

quantitative and qualitative description of parameters associated with morphological aspects, which correspond to patterns in the images by appropriate scaling. Other applications of DIQA are: in astronomy, images of radiotelescopes are used for determining the relative distances of the stars; in geography, from satellite images, DIQA is used for the description of the water system of a region; in neurosciences, by various techniques of microscopy, images are used to measure the perimeter and the length of dendritic tree of neurons; and, in medicine, from magnetic resonance imaging and by employing different chromophores agents, images are used for the detection of specific molecules in biological systems [1-3].

On the other hand, the color is a subjective characteristic of substances and objects, therefore, the correct measure for quantitative purposes must ensure the reproducibility under the same test conditions. At present, the capturing of a digital image is made commonly in the day-to-day, but the perception of the colors present in it will depend on the team capture the image, environmental conditions, image projection device and the features of the sample [4-7].

In particular, a digital camera uses a standard format for computers for the storing of information obtained by a CCD (Charge Coupled Device); the CCD is a light sensitive sensor, consisting of millions of small semiconductor silicon capable of capturing photons and generate an electrical response that subsequently is converted into digital data that constitutes a pixel, which in turn constitute each point into the image [3]. It is in this pixel, therefore, where the color information that can be analytically useful is stored. These data can be used for analytical applications since these are not subjective in nature (although its projection on a monitor or photograph follow being subjective in nature). Consequently, under analytical approaches, a device for the making of a digital image is a capture instrument of electromagnetic information emitted by the sample in the visible region of the electromagnetic spectrum. Thus, it is clear that the quality of such information and the ability of its quantitative interpretation are the determinants of appropriate quantitative application.

The aim of this work was to develop and evaluate a quantitative parameter that allows the description of the color intensity of analytical samples for application in DIQA; in addition, we show its

application in the construction of the McFarland scale for determination of colony forming units (CFU) in the microbiological analysis.

2. Theory

2.1 RGB space

RGB space, or Red-Green-Blue space, is the most common model used in electronic and computer for the storing of color information in digital formats. This model defines three primary colors (red, green and blue) for the construction of a color space that mimics human vision and with which the information is encoded in one pixel. The theory and fundamentals of the RGB model and its application to different technologies have been documented [8-9]. In the RGB model, any color is a vector resulting from the sum of three primary vectors, $\mathbf{R} = (255, 0, 0)$, $\mathbf{G} = (0, 255, 0)$ and $\mathbf{B} = (0, 0, 255)$, where elements of all vectors are integers between 0 and 255 (or between 0 and 1) [8-10]. In electronic devices, the color information is captured and stored in a digital format by "bits" using the binary system. Later, these bits are used in the building of a 2D image formed by thousands of bits. Thus, for a single bit, any color is defined in the RGB space by a vector (ϕ_0) whose magnitude can be described by:

$$\phi_0 = \sqrt{R^2 + G^2 + B^2} \quad (1)$$

where R , G and B are the magnitudes of the vectors $\mathbf{R}$, $\mathbf{G}$ and $\mathbf{B}$, respectively (see Figure 1).

Figure 1. Illustration of RGB space

2.2 Index surface color

To construct a quantitative parameter for the objective evaluating of color variations, from the color information captured by digital devices, the color unit (u_c) is defined as the inverse of the magnitude of the vector resulting from the contributions vectors:

$$u_c = \frac{f_m}{\phi_0} \quad (2)$$

where f_m is a magnification factor used in order to increase the numerical value u_c ($f_m = 1 \times 10^4$) [11]. Because the captured information is encoded 2D images, u_c is not appropriate since it corresponds to the color stored in one bit. For image analysis, a surface is delimited and analyzed, in consequence, a second parameter, called surface color index (I_{cs}) is defined as the average u_c in a selected area of the image. Thus,

$$I_{cs} = \frac{f_m}{n^2} \left[\left(\sum_{i=1}^n R_i \right)^2 + \left(\sum_{i=1}^n G_i \right)^2 + \left(\sum_{i=1}^n B_i \right)^2 \right]^{0.5} \quad (3)$$

where n is the number of pixels of the analyzed surface, which is the same in all cases. Thus, a calibration curve can be made and associated with I_{sc} values as a function of the concentration (C) or any other parameter associated with the color. Thus, for a linear behavior:

$$I_{cs} = m_1 C + m_2 \quad (4)$$

where m_1 and m_2 are the slope and intersected resulting from correlation analysis, respectively. In addition, an amplification factor (F) is introduced in order to increase the distance between the analytical response for values of consecutive concentration (F modifies the slope of the line without altering the intersected and the scale of the independent variable). Thus we have that

$$I_{cs}^* = F I_{cs} - m_2 (1 - F) \quad (5)$$

I_{cs}^* where is the amplified response. Note that in Equation 2, the analyzed surface is implicitly

assumed to be a set of points with the same value of u_c and in consequence: (1) an ideal surface for color can be defined, *i.e.*, a surface whose bits have a unique I_{cs} value, and therefore a zero standard deviation, and (2) the description of the interfaces by detecting changes I_{cs} can be carried out. However, for real surfaces it is assumed that I_{cs} values are randomly distributed, and a non-random distribution corresponds to two completely differentiable phases by color.

3. Experimental section

3.1 Design and construction of the measuring device

An image of the measuring instrument and its main components are shown in Figure 2, this was built by the GI-CAT of Universidad del Valle and Mindtech-RG of Mindtech s.a.s. The main components of the measuring instrument are: (a) An image capture device (Nikon Coolpix P530 digital camera model with a 42x optical zoom), (b) a cell sample holder (glass cell for spectrophotometry 45x12.5 x12.5 mm, 10 mm pitch.), (c) a surface to set a background color (aluminum foil coated SiO_2 for different colors to white, the white surface of SiO_2 was coated with black paper not bright), (d) a cabin for analysis with an internal lighting system (Lighting devices were placed around the sample holder), (e) a computer for processing information and (f) a reading software I_{cs} to from the captured image (Spectrum 2.0, Mindtech s.a.s., Colombia).

Figure 2. Measuring instrument for capturing images.

Tabla 1. Dyes used as test sample (RB-129 is Reactive blue 129).

Dye	Color	Vector RGB asociado	Code
Reactive Red 129	Red	(1,0,0)	RR-129
Reactive Yellow 145	Yellow	(1,1,0)	RY-145
Acid Blue 129	Blue	(0,0,1)	AB-129
Acid Black 48	Black	(0,0,0)	AB-48
Mixture 1:1 de RB-129 y RY-145	Green	(0,1,0)	RB-129+RY-145

Figure 3. Chemical structure of dyes used and their respective color representation.

3.2 Test samples

To evaluate the analytical capacity I_{cs} , for the quantitative description of color, different textile dyes solutions of known concentration (2-100 mg/L) were used. The dyes used are shown in Table 1 and their respective structures are shown in Figure 3. These dyes are selected to include the primary colors of the RGB space and two additional colors, associated with grayscale (black) and a secondary color in the RGB space (yellow). The solutions were prepared in triplicate.

3.3 Light homogeneity test

To ensure that the conditions of internal light do not alter the perception of homogeneity of the solution and color (by shadows or reflections flashes), a verification of the homogeneous distribution of color was made. For this, a test image was captured using a dye test (AB-129); subsequently the I_{cs} for a total of five subsamples within the region analyzed was measured. Comparisons I_{cs} values were performed by analysis of mean value, standard deviation and coefficient of variation.

3.4 Evaluation of analytical parameters

The limits of detection and quantification, LOD and LOQ, respectively, were determined by the performing of measurements using distilled water. The accuracy and precision were evaluated from two samples with different concentrations (15 mg/L and 60 mg/L) for each dye.

3.5 McFarland scale and CFU determination

To determine the CFU, it was performed microbial growth experiments by dispersing in a liquid medium of Gram negative bacteria (*Escherichia coli*), using soy broth culture trypticase 30 g/L (Aldrich). To standardize the concentration of inoculum in CFU/mL, the turbidity of the broth was measured UV-vis spectrophotometry at 625 nm using a Shimadzu UV-1700. For this purpose, a suspension of barium sulfate is prepared from sulfuric acid (0.18 mol/L) and barium (0.048 mol/L) chloride [10, 12]; thus, a calibration curve was made in the range of 0.5 to 10.0 CFU/mL (McFarland scale). Then, 100 μ L of the bacterial dispersion were inoculated into 7.0 mL of broth previously prepared and sterilized. Finally, the systems were incubated at 37 °C for 24 hours. After this time, bacterial growth was measured by UV-vis at 625 nm. Different control tests were performed for the *E. coli* growth in the presence of starch (positive control, *i.e.* growth should be noted), ampicillin (negative control, *i.e.*, absence of bacterial growth), and two blank experiments: with distilled water (analytical blank) and using tryptic soy (bacterial blank).

4. Results and discussion

4.1 Lighting homogeneity test

The vector components of RGB space obtained for the different subsamples, during lighting homogeneity test, are shown in Table 2. The coefficient of variation obtained was 0.8% with average values of I_{cs} 356; therefore, the results showed that internal light conditions are homogeneous, with fluctuations in the color intensity of ± 3.0 units of I_{cs} . This indicates that there are not internal reflections or light flashes and, therefore, variations between adjacent pixels can be attributed to random distributions of color.

Table 2. Vector components of RGB space and values of I_{cs}

Height	R	G	B	I_{cs}
5	168	160	162	353.4
4	167	159	162	354.9
3	165	157	160	359.3
2	165	157	160	359.3
1	150	143	156	385.5

Table 3. Correlation parameters for the test solutions

Dye	Correlation parameter			
	m_1	m_2	R^2	Obs.
RR-129	0,176	44,09	0,997	L
AB-129	0,194	46,59	0,996	L
RB-129 + RY-145	0,086	43,89	0,995	L
RY-145	0,030	50,86	0,995	L
AB-48	0,020	42,41	0,997	Exp.

R^2 = Correlation coefficient. For linear and exponential expressions, equations are $I_{cs} = m_1C + m_2$ and $y = m_2\exp(m_1C)$, respectively. L = linear behavior. Exp. = Exponential behavior

Correlation parameters obtained for each test sample are shown in Table 3. Appropriate linear correlations were observed for the solutions of red, blue, yellow and green colouration, being possible to construct calibration curves over a range of 2 to 100 mg/L of dye. The respective calibration curves are shown in Figure 4A (Average correlation coefficient was 0.996).

In the case of AB-48, an exponential behavior was observed, therefore, Equation 5 can not be applied without a previous linearization (see Figure 4B). In particular, the reduced concentration of AB-48 causes the observed color change from black to white, *i.e.*, the observed variation occurs along a specific direction in the RGB space (gray scale).

4.2 Limits of detection (LOD) and quantitation (LOQ)

Values of LOD and LOQ, which are defined to be three and ten times the standard deviation of blank experiment, respectively, are parameters associated with the perception of that color when the liquid inside the glass cell is only water. However, although the water is translucent, the camera detects a value of $I_{cs} = 45.6 \pm 3.3$. This value is explained as the result of the image associated with the glass cell. The LOD and LOC values were 9.9 and 33.2, respectively.

4.3 Accuracy and precision

Mean values and standard deviation for the measure of the two dye samples (15 and 60 mg/L) are shown in Table 4; likewise, the regression parameters for

respective calibration curves also are shown. The average relative error percentage was 9.1% and the average standard deviation was 3.16 mg/L. In addition, the average coefficient of variation was 13.7%.

Figure 4. I_{cs} calibration curves versus dyes concentration for linear (A) and exponential response, AB-48 (B).

Table 4. Average and standard deviation of two dye samples with known concentration (15 and 60 mg/L)

Sample	Conc. (mg/L)	Correlation parameter			m_1 (L/mg)	m_2	R^2
		Average Conc. (mg/L)	Stdr Desv. (mg/L)				
RR-129	15	12.2	3.6		44.09	0.997	0.994
	60	55.3	3.4				
AB-129	15	14.8	3.4		46.59	0.996	0.998
	60	60.2	3.8				
RB-129 + RY-145	15	16.0	2.5		43.89	0.995	0.990
	60	62.3	2.6				
RY-145	15	16.7	3.3		50.86	0.995	0.994
	60	63.5	2.1				
AB-48	15	14.8	3.4		42.41	0.997	0.994
	60	56.1	3.5

Figure 5. Scale McFarland to assess CFU during growth of *E. coli*

Figure 6. I_{cs} and Mcfarland scale (6A) and correlation measurement I_{cs} regarding measures by UV-vis spectroscopy (6B).

4.4 McFarland scale and UFC determination

The McFarland scale is based on turbidity generated by the suspension of bacterial colonies in aqueous media, it establishes a direct relationship between the UFC and the degree of turbidity. Usually, the turbidity is measured by spectrophotometric techniques through an acceptable linear behavior. In our case, the measurements by UV-vis spectroscopy allowed the construction of calibration curves with an R^2 of 0.996 (for dispersions range from 3×10^8 to 3×10^9 CFU); the respective regression equation was $I_{dr} = 5 \times 10^{-10} \text{ UFC} + 0.0807$ (where I_{dr} is the intensity of dispersed radiation).

Furthermore, measurements I_{cs} were made to McFarland standards (see Figure 5, the red box shows

the measures that showed a linear fit). By use of a black-color background, an opposite trend to that obtained for white-color background, and an exponential decay curve can be linearised satisfactorily to concentration values of 0.6 mg/L.

Thus, it can be observed that, for a value of $F = 1$, the value obtained of R^2 was 0.9919 where the regression equation is given by $\ln(I_{cs}) = -0.6868C + 3.5765$ (see Figure 6A); however, for a value of $F = 10$ is obtained $\ln(I_{cs}^*) = -6.868C + 3.5765$. Also, when the degree of correlation between the methods are compared, spectroscopic method and DIQA, it is observed that the absorbance correlates satisfactorily with I_{cs} between 0.0 and 1.0 mg/L (see Figure 6B). The equation of respective correlation was $I_{cs} = -0.4544\text{Abs} + 3.5969$ ($R^2 = 0.9986$).

The results of the CFU determination in five experiments are shown in Table 5 (these experiments were using only water, trypticase soy, ampicillin, starch and bacteria and were named as MH₂O, MTS, MAMP, Malm and MEcoli respectively).

Table 5. CFU for test experiments

Experiment	Results		
	I_{cs}	UFCx10 ⁶	Description
MH ₂ O	56.0 ± 0.5	8.9	No growth
MTS	50.9 ± 1.4	9.2	No growth
MAmp	38.8 ± 1.1	9.9	No growth
MAlm	33.6 ± 0.9	10.3	Growth
MEcoli	33.7 ± 0.6	10.3	Growth o

An important aspect in these experiments is that the result can be predicted and experimental measurements can be easily evaluated. Thus, because it was used a black background, the respective vector in the RGB space will close coordinates (0,0,0), and taking into account the definition given in Equation 2, it is expected that the maximum value I_{cs} in experiments must correspond to MH₂O. As microbial growth is not present, the solution will be more transparent and therefore the expected value is expected to be close to the maximum value (samples where no growth is expected are MTS and Mamp).

Finally, because the starch hasn't antimicrobial activity, it is expected the bacterial growth and a decrease of I_{cs} . The same result is expected for MEcoli. As it can be seen, the results were completely consistent. The experimental order of I_{cs} values was MH₂O > MTS > Mamp > Malm ~ MEcoli. It can be seen that although the experiments MH₂O, MTS and Mamp have minimum values I_{cs} , the calculated UFC value are different from zero. This is explained by the presence of color in the culture medium, suggesting that the presence of color in itself does not alter the relative result but the comparisons of numeric values since all samples are prepared under the same conditions.

5. Conclusions

The I_{cs} based on the RGB model that can be used for the quantitative description of color by digital image analysis using controlled conditions. This allows the

construction of calibration curves with a measuring range up to 100 mg/L. The measurement conditions for the capture of image must be calibrated and evaluated for each type of experiment to ensure the absence of environmental interference, being useful for this the use of a lighting homogeneity test.

The digital image quantitative analysis can be used to determine CFUs under different measurement conditions. In this regard, the method described here acts as a quantitative analysis method and it can be used for the replacing of techniques such as spectroscopy or turbidimetry.

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Interest conflict

No interest conflict

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