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Development and testing of multi-well plates absorbance reader for clinical analysis using inexpensive Web-Cam

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ABSTRACT

Biochemical analysis and clinical tests like glucose, hemoglobin, cholesterol, iron, etc. are crucial for early illness diagnosis like diabetes, anemia and coronary deceases. These tests usually are done in state of the art instruments in well equipped laboratories in health centers. In some cases, these instruments are not portable, so they are not recommended for clinical field studies in remote areas. The present work shows a portable low-cost prototype of multi-well plates reader designed for clinical analysis. A Light Emission Diodes (LEDs) array is used as excitation source and an inexpensive webcam as detector. The light source illuminates the 96 well plates and the webcam take the image with 640x480 pixels. The data is acquired and processed by using a portable computer. 96 samples can be read including blanks and calibration standards simultaneously. Light absorption data are processed using a MatLab software designed in our laboratory to obtain calibration curves, standards lectures and samples concentration. The system was evaluated using different analytes series solutions: Neutral Red, Cooper (II) Ammonia Complex and Methyl Orange. The results shows that it is possible to measure few micro liters of solutions with adequate exactitude and precision of less than 3%. As possible analytical clinical application, iron determination was performed using Fe(III) Thiocyanate complex. This method is usually applied in serum samples analysis. The sensibility achieved with the proposed instrumentation configurations allows the analysis of iron in serum samples in the references values normal range (0.75 – 1.5 mg/L) in human.

Keywords: Spectrophotometry, Colorimetric assays; Bioassays; CCD camera.

1. INTRODUCTION

The technology of digital image acquisition such video cameras base on charge-coupled-devices (CCD), commonly knows as WebCam, allows to capture digital image with high bits resolution that is traduced in millions colors. By using the RGB (Red-Green-Blue) color system, these primary colors can be combined in different intensity to produce any color with value varying in the range at least of 0-255 (8 bits) per color.

The high spatial resolution of the digital images, that allows the modern digital cameras facilitates the use of extremely small observation areas onto the monitored samples, which can leads to a) save on expensive or contaminant chemical compounds, employed in chemicals reactions for color develop, 2) the use of small samples container and 3) simultaneously multi-samples and/or calibration standard measurements.

The images acquired by using CCD digital camera have been applied for different spectroscopy techniques, especially in colorimetric test. The application of digital imaging as detector for colorimetric test has great potential for many applications which involve the production chemical color changes such: absorption spectrum variation or intensity color variation associated with analyte concentration variation, both in qualitative and quantitative application for chemical compound with strong visible absorption.

Digital cameras can act as analytical detector, allowing to generate a vast amount of information per image frames that contain an analytical information of high number of samples and calibration standard allowing all calibration standard

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and samples data can be obtained by taking a single image and this means or conduce to a sensible decrease in the analysis time compare to those necessary with conventional spectrophotometer instruments.

Different author report the applications of web cameras as spectroscopy detectors, such instruments can be sufficiently sensitive to detect the color changes of different substance. More information can be acquired using computers screens as light sources referred as computer screen photo-assisted technique (CSPT). This technique has been applied in different kinds of analytical application such: bioassays, food analysis, etc[1-6].

Most ultraviolet-visible (UV-VIS) applications are based in measuring the intensity of the absorption of relative monochromatic radiation across a range of wavelength passing through a solution in a cuvette. The practical wavelength region extends from 190-400 nm (UV range) and from 400-780 nm (VIS range). In a typical experiment, a light beam of intensity (I_o) strikes a sample consisting of a quartz o glass cell containing a solution. After passing through the cell, the light beam has reduced intensity (I) due to analyte absorption. The intensity of an absorption band, absorbance $A=log(I_o/I)$, is proportional to the number of absorbing species in the illuminated part of the sample cell according Beer's law.

In general the classic UV-VIS spectrophotometer is consisting of these main components: a broad spectrum light source, dispersive unit (monochromators), sample cell, detector, electronics and computer for data manipulation and storage. Depending on the dispersive-detector devices characteristic, the instruments can be monochannel or multichannel. The mono-channel system only one detector is used. In the multi-channel an array detector is used.

The photometers are the most popular instruments in clinical laboratory, especially in non well-developed areas where more sophisticated and expensive instruments are prohibitive. In photometers, the expensive monochromators units are replaced by one or more interference filters, thus making system usable for specific applications at given wavelength especially in visible region. The photometer is a mono-channel instrument, it measured the absorbance for each sample and/or standard one by one that is time consuming, specially when a large amount of samples and test have to perform.

Well-know colorimetric reactions are used for clinical analysis such as urea, glucose, enzymes, cholesterol, some metals in serum, blood and others fluids. Quantitative determinations are based in absorbance measurements in samples where the analyte form specific strong visible complexes.

The main purpose of this works to use a inexpensive web-cam ordinary used for internet communication, for quantitative analysis of different organic and inorganic analytes that can be transformed by means specific chemicals reactions in strong colored compounds, specially to those associated in clinical test. For future low-cost and portable clinical instruments production for remote areas in no-developed countries applications.

2. EXPERIMENTAL

2.1 Instrumentation

Figure 1 shows a schematic drawing of the new WebCam spectrophotometer. A 2D Array light emission leds (LEDs) used as light source illuminate a 96-well microplate placed in removable holder. A diffuser screen was used for the homogenization of the microplate illumination. A CCD WebCam 640 x 480 pixels (GE 98067 MiniCam Pro) placed collinearly with the light source obtain the image of the plate. The samples are placed in conventional transparent plastic microplates with 96 wells each one with 360 micro liter volume capacity. The microplate is supported onto an automatic drawer. The rest of the setup is a black box that shields the measurements from ambient light and provides support for the webcam. The latter is connected to a laptop computer for images acquisition and processing. This detector use a diagonal 4.5mm (Type 1/4) interline CCD solid-state image sensor with a square pixel array which supports VGA format. Progressive scan allows all pixels signals to be output independently within approximately 1/30 second. Also, the adoption of monitoring mode allows output to an NTSC monitor without passing through the memory. This chip features an electronic shutter with variable charge-storage time which makes it possible to realize full-frame still image without a mechanical shutter. High resolution and high color reproductivity are achieved through the use of R, G, B primary color mosaic filters. Further, high sensitivity and low dark current are achieved through the adoption of HAD (Hole-Accumulation Diode) sensors[7]. Figure 2 show the CCD spectral sensitivity characteristic.

2.2 Data analysis

The light transmitted from the microplate creates an image of the array under illumination, which is subsequently acquired by the web camera operating at a capture rate of 15 frames per seconds during a specific integration time. The result is a digital video file (AVI format) of the array under illumination. The video is decomposed in their individual frames and the information contained in the wells is extracted. A digital mask is used to take out the information from circular areas centered on the image of the wells, which avoids the borders where the meniscus formation might introduce a spurious reflectance.

The digital values of each primary color level (RGB) of the pixels composing the image are average giving the intensity per color channel for each well. All the wells in the frames are evaluated in the same way. Finally, the average intensity of each well is integrated during the exposition time.

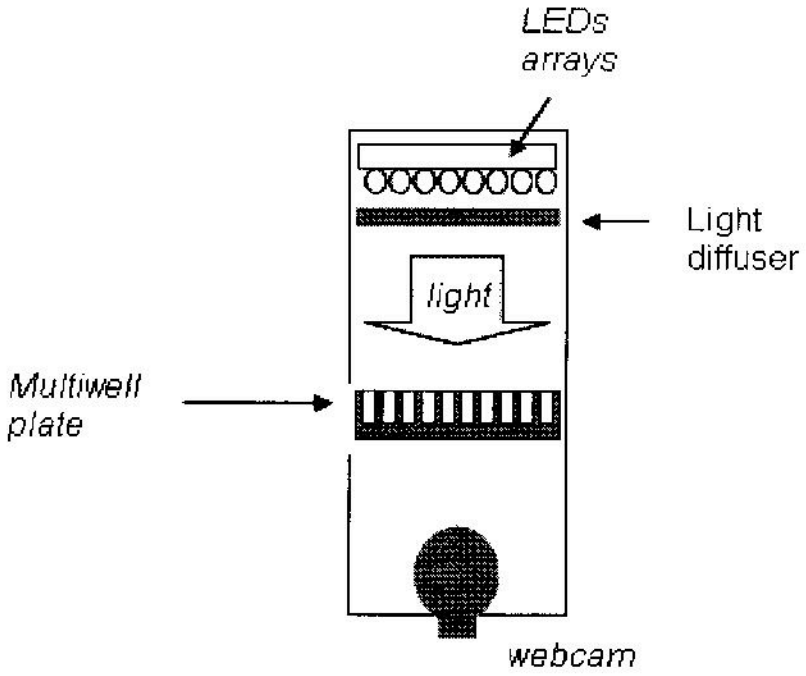

Figure. 1. Schematic of the experimental set-up for absorbance measurement in multi-well plates using a LEDs arrays as light source and webcam as detector.

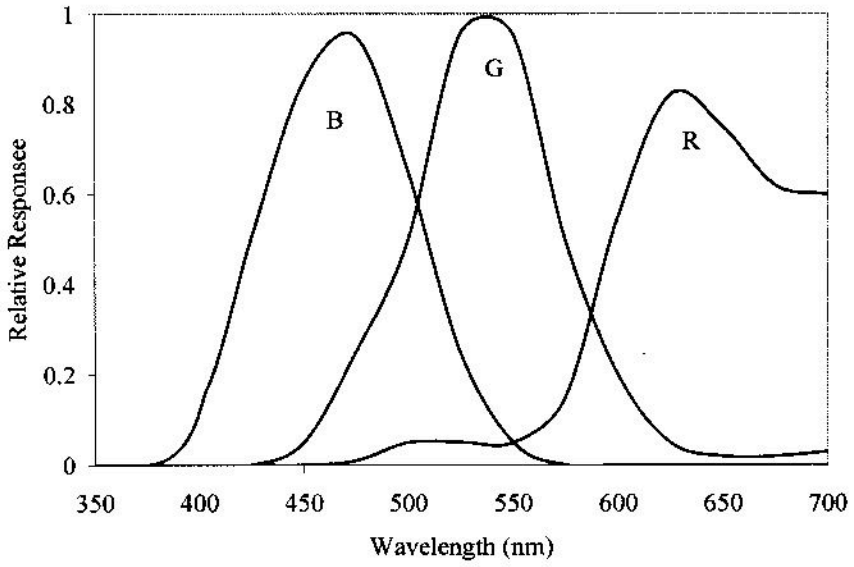

Figure 2. CCD spectral sensitivity characteristic (includes lens characteristics, excludes light source characteristics).

In all case wells containing a blank solution are measured. The transmitted intensity of each well, containing samples, standards or blanks are obtained for each primary color allowing the absorption calculation by using:

$$A^i = \log \frac{I_0^i}{I^i} \qquad (1)$$

Where: A is the absorbance, I_0 and I are the integrated intensity transmitted by the blank and the samples respectively, the superscript i indicates the primary color (RGB) at which the evaluation is made. Calibration curves for standards solutions are constructed by using the measured absorbance at three primary colors (A^R, A^G and A^B).

All data are processed using a MatLab software designed in our laboratory obtaining calibration curves, standards lectures and samples concentration.

The proposed system was evaluated with different solutions with absorption spectra covering the visible range: neutral Red, methyl orange, cooper (II) ammonia complex, and iron thiocyanate complex that it is usually used for iron determinations in serum samples. A series of aqueous solutions were prepared in appropriate concentration range. Absorbances were measured using a conventional spectrometer (Ocean Optics USB 2000) for comparative purposes.

3. RESULTS

3.1 Samples Absorption Spectra

Figure 3 shows the absorption of the neutral red, methyl orange, ammonia-cooper (II) and iron-thyocianate complexes recorded using conventional spectrophotometer. The wavelengths for absorption maxima for each compound are: 620, 540, 478 and 470 nm for cooper-ammonia complex, neutral red, iron-thiocyanate and methyl orange respectively. With those compounds solutions the visible range of absorbance it is entirely covered (from 375 to 650) in order to evaluate both: the LEDs as excitation source and the WebCam as light transmission detector.

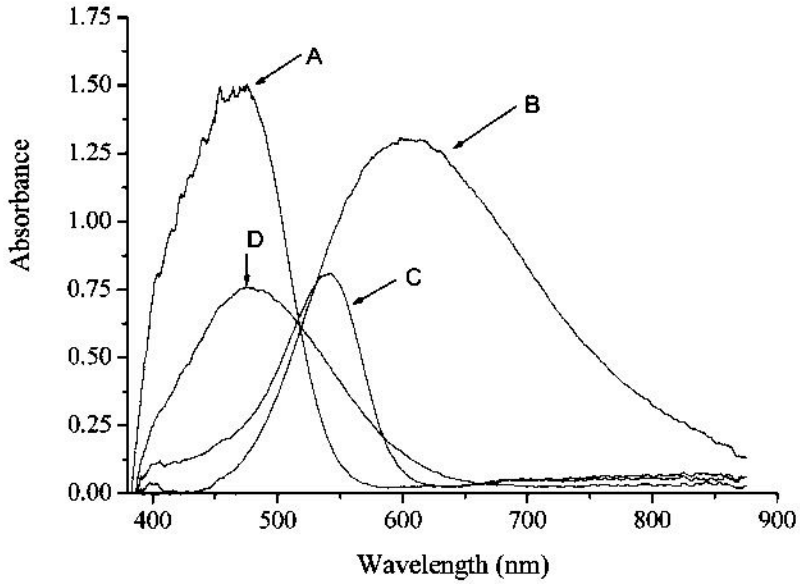

Figure 3. Absorption spectra for Methyl orange (A), Copper-ammonia complex (B), Neutral red (C) and Iron-thiocyanate complex (D) obtained using conventional spectrometer.

3.2 Calibration Curves

Calibration curves were performed for each standards solutions series. Dispensing 180 µl solution volume en each micro-plates wells. Figure 4 shows the calibration curve for neutral red using absorbance signal measured on the three CCD Channel (R,G,B). It is clearly notice that a linear behavior is only obtained with green channel absorbance signal. For red channel a signal independent from concentration is observed. On other hand, blue channel signal increases with concentration in the range from 0 to 4 mg/L and then saturates. Theses result are according with the neutral red absorption spectra (figure 3) that shows a maximum at 540 that it is included in the range of the green channel. The spectral band is extended until 400 nm but with lower intensity. This effect can explain the behavior of the signal detected in the blue channel. The neutral red has an absorbance close to cero for wavelength higher than 600 nm that correspond to the range of the red channel.

In figure 5 shows the calibration curves for methyl orange using absorbance signal measured on the three channels. In this case, the linear behavior for absorbance signal as a function of sample concentration is obtained only for the signal in the blue channel. For red and green channel a signal independent from concentration is observed. These results are according with the absorption spectra (figure 3) that shows only one maximum at 470 nm that it is centered in range of the blue channel.

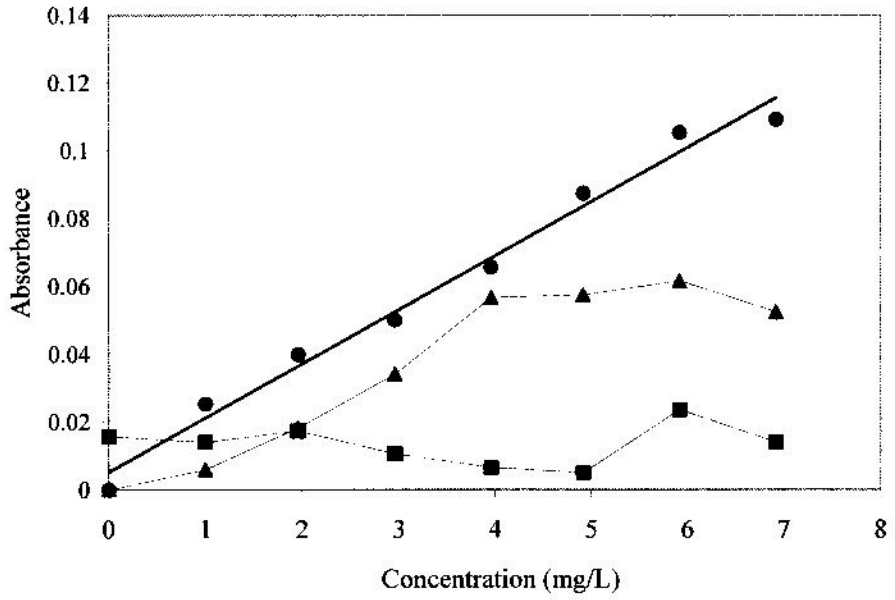

Figure 4. Calibration curve for Methyl Red obtained with red channel (■), green (●) and blue (▲) absorbance measured signal.

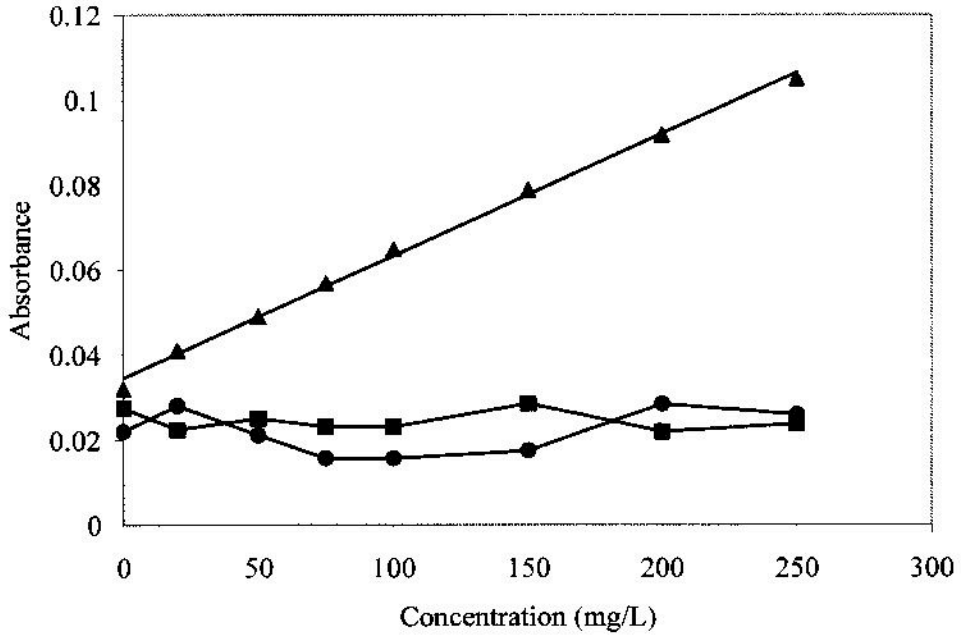

Figure 5. Calibration curve for Methyl orange obtained with red channel (■), green (●) and blue (▲) absorbance measured signal.

Similar experiments were performed with all compounds obtaining that the better channel for absorbance measurement for calibration purpose; correspond to those that include the absorption maximum. For the copper-ammonia complex the red channel is selected. For the iron-thiocyanate the blue channel is selected.

Figure 6 shows a calibration curves obtained using conventional spectrometer and the proposed WebCam instrument for red neutral solutions, using the same standards solutions. A dramatic diminish in the sensibility (calibration curve slope) is observed for the WebCam instrument compare to those obtained with conventional spectrometer. It is explained due to absorbance is made using a polychromatic radiation selected by the CCD camera (green channel) instead a relative monochromatic radiation ($\Delta\lambda = 1$ nm) provided by the spectrometer. Even though, the WebCam instrument can be used for a wide range of applications, where high sensibility is not required.

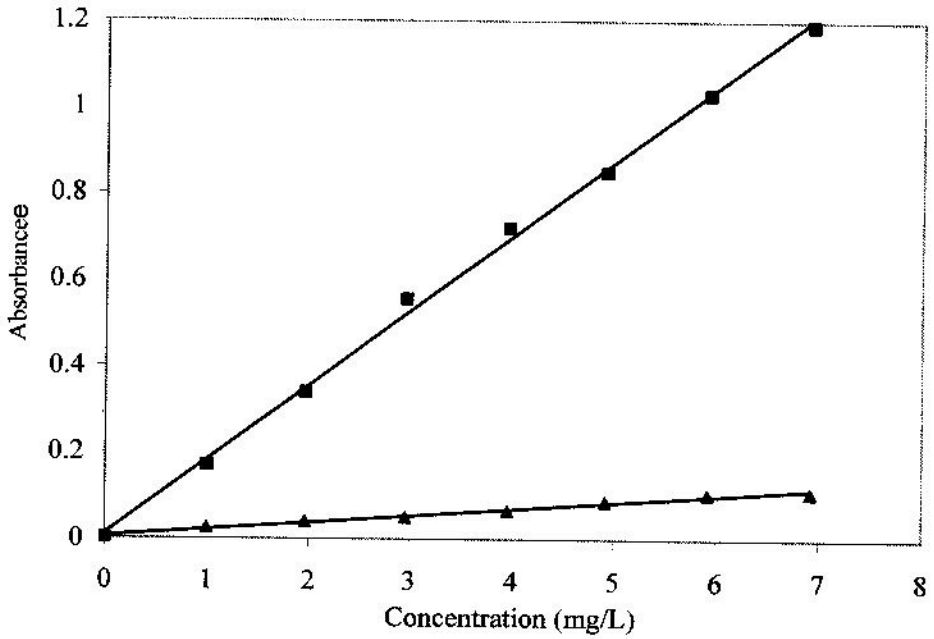

Figure 6. Calibration curves for Neutral red obtained with conventional spectrometer (■) and the proposed WebCam instrument (▲).

3.3 Iron Quantitative Analysis

As possible analytical clinical application, iron determination was performed using Fe(III) Thiocyanate complex. This method is usually applied in serum samples analysis. The absorption spectrum for the Iron-thiocyanate complex shows a maximum at 470 nm (see figure 2). The blue channel was chosen for absorbance measurement. Table 1 shows the calibration parameter of the linear fitting using least squares. It is clearly notice that good calibrations curve was obtained. In order to increase the sensitivity, two times the volume (360 μ liter) of each standard were dispensed in each micro-plate well. In this case, a significant enhancement of sensitivity (near twice) was obtained with the increase of the sample thickness.

Table 1. Calibration curves obtained for Fe(III)-thiocyanate complex

Sample volume	Concentration range ($\mu g\ ml^{-1}$)	Slope	Intercept	Correlation coefficient (R^2)
180 μ liter	0 to 3	0.0274	0.0035	0.9949
360 u liter	0 to 3	0.0540	0.0057	0.9946

The proposed instrumental configuration was also applied in the determination of total Iron in some reference samples. four samples were tested and their replications. The results are shows in table 2. In all case not statistically difference are found between the predicted and real concentration values at the 95% confidence level. The good agreement between these results and known values indicates the successful applicability of the proposed instrumental configuration for Iron analysis using thiocyanate complex. The sensibility achieved with the proposed instrumentation configurations allows the analysis of iron in serum samples in the references values normal range (0.75 – 1.5 mg/L) in human[8].

Table 2. Estimated and actual iron concentration in references solutions.

Sample identification	Fe concentration ($\mu g\ ml^{-1}$)	
	Actual	Found
1	1.20	1.16 ± 0.03
2	1.50	1.47 ± 0.02
3	2.50	2.45 ± 0.05
4	2.70	2.66 ± 0.07

4. CONCLUSIONS

The feasibility of the use of digital images obtained from WebCam as direct instrumental absorption detector for quantitative analysis was demonstrated.

The digital primary colors filters R, G, B were used as wavelength selectors in order to evaluate the absorbance at the maximum analyte's absorption spectra. In contrast to conventional spectrometer, a dramatic diminish in the sensibility (slope of the calibration curve) is observed in the WebCam instrument. However, this new instrument can be used in any applications, where high sensibility is not required.

The proposed instrumental configuration was successfully applied in some colored substances with strong visible absorption like total iron determination. ,

The system can be built with economics parts: LEDs for as light source, ELISA microplates as samples container and WebCam as detectors. This system can be considered as a promising analytical too for accomplishing quantitative chemical analysis, as well as it offer an economically viable alternative for clinical analysis.

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