Talanta 126 (2014) 145–150

Contents lists available at ScienceDirect

Talanta

journal homepage: www.elsevier.com/locate/talanta

Short communication

A colorimetric microwell method using a desktop scanner for biochemical assays

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ARTICLE INFO

Article history:
Received 31 January 2014
Received in revised form
25 March 2014
Accepted 27 March 2014
Available online 2 April 2014

Keywords:
Biochemical analysis
Scanner
Colorimetric sensor
Graphic User Interface

ABSTRACT

A method for rapid, inexpensive and sensitive simultaneous analysis of glucose, creatinine, triglycerides, total cholesterol and total protein is needed to analyze blood. The proposed method is based on the production of a specific color after reaction. The method was adapted to a 64-microwell plate format, and it uses the transparency scanner feature of a commercially available desktop scanner. Each microwell plate had an 8×8 array of flat-bottomed 250 μL microwells, and these microwells were used to simultaneously house the solutions for clinical assay. The scanned image was saved in TIFF format in a portable computer and then processed using a Graphic User Interface (GUI) designed in our laboratory to obtain analytical curves and to automate the mathematics and statistics calculations. This automation improved the analytical frequency of the method. The results showed that it is possible to measure a few microliters of solution with exactitude and precision better than 5.30%. The measured concentration ranges of glucose, triglycerides, creatinine, total cholesterol and total protein were 0.781 to 100, 1.56 to 200, 0.031 to 4.0, 1.56 to 200 mg dL^{-1} and 0.031 to 4.0 g dL^{-1}, respectively. The limits of detection were 16.2, 51.7, 0.12, 41.5 mg dL^{-1} and 0.62 g dL^{-1} for glucose, triglycerides, creatinine, total cholesterol and total protein, respectively. The recoveries were from 98.7% to 101.3% for total cholesterol, 98.7% to 124.9% for triglycerides, 54.2% to 98.3% for total protein, 89.6% to 101% for glucose and 65.7% to 115.4% for creatinine. The results provided by the scanner were compared with those obtained with a commercial photometer and did not show significant differences at a confidence level of 95%. Good results were obtained for the correlation coefficient and Root Mean Square Error of Prediction (RMSEP) values for the five parameters, especially the total cholesterol and creatinine. The RMSEP values for glucose, creatinine, triglyceride, total cholesterol and total protein were 8.05, 0.28, 7.69, 1.41 mg dL^{-1} and 2.2 g dL^{-1}, respectively.

1. Introduction

Biochemical analysis and clinical tests are crucial for early diagnosis of illness (diabetes, anemia and coronary diseases). The vast majority of the biochemical analyses are based on enzymatic methods [1] that produce a specific color after reaction. For example, total cholesterol is determined by a colorimetric enzymatic assay with cholesterol oxidase. The final product is the red-colored quinine imine ($\lambda = 500$ nm) whose color intensity is proportional to the original concentration of the total cholesterol in the sample. Quantitative determination for other biochemical assays, such as glucose, total protein, total cholesterol, triglycerides and creatinine, is based on absorbance measurements where the analyte also forms specific complexes that absorb in the visible portion of the electromagnetic spectrum. For biochemical laboratory measurements, quantitative methods must be accurate and precise over the range of required values.

The concentration of blood glucose is an indicator of two common diseases: hypoglycemia and hyperglycemia [2]. Total protein is the most abundant compound in our serum, and its concentration is associated with many diseases, such as chronic infection, hemolysis and cyroglobulinemia. Additionally, a low concentration of total protein can indicate pregnancy [3]. The concentrations of total cholesterol and triglycerides are intrinsically related to heart diseases [4,5]. Creatinine is a nonenzymatic compound derived from creatine (methyl guanidoacetate), and its concentration is widely used to identify renal disease [6,7].

Clinical tests of these parameters performed in laboratories with low infrastructure and technology, as in non-developed countries or remote zones, are time consuming and demand large

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http://dx.doi.org/10.1016/j.talanta.2014.03.066
0039-9140/

volumes of reagents. Commonly, these tests are semi-automated and just one analysis is performed at a time. However, the use of image processing can help automate these procedures, reducing the time spent in analysis, the volume of reagents, the amount of human blood required and the equipment costs.

Optical scanners combined with chemical sensors, mainly as sensor arrays, are interesting analytical options for use in the research, industrial and occupational fields. Several standard colorimetric reactions for chemical analysis have been miniaturized to be used with microwells on microplates. These include methods that use computer screen photo-assisted techniques (CSPT) [8,9]. The advantages of miniaturization are reduced amounts of reagents, improved safety, reduced waste and increased sample throughput. In addition, the technology of digital image acquisition, such as video cameras based on charge-coupled-devices (CCD), enables the capture of digital images with high resolution. These images are translated into millions of colors, simultaneously leading to multi-sample and/or standard calibration measurements. By using the RGB (red–green–blue) color system, the RGB primary colors can be combined to produce any color array, generating a colored image. The intensity values for each RGB channel vary in the range of 0 to 255 (8 bits).

Digital image analysis can replace a classical spectrophotometer for quantitative colorimetric analysis, as has been shown for colorimetric methods for the microscale determination of ammonium [10], bromide [11], nitrate [12] and phosphate [13,14] ions. Herein, the main purpose of this work was to use a 64-microwell plate format and a desktop scanner for quantitative biochemical assays (glucose, creatinine, triglycerides, total cholesterol and total protein) in blood using a two-dimensional (2D) array for home tests.

The present paper proposes a method for rapid, inexpensive and sensitive simultaneous analysis of glucose, creatinine, triglycerides, total cholesterol and total protein in blood samples. The method was adapted to a 64-microwell plate format, and it uses the transparency scanner feature of a commercially available desktop scanner. In addition, a Graphic User Interface (GUI) designed in our laboratory to obtain analytical curves and to automate the mathematics, statistics calculations and improve the analytical frequency of the method.

2. Experimental

2.1. Samples

Experiments were performed with synthetic and real samples. Synthetic samples consisted of the regent that contains the immobilized biochemical compound. All the chemicals were acquired from Bioclin® (Quibasa Química Básica Ltda, Brazil) [15]. For total cholesterol, creatinine, glucose, total protein and triglycerides, the reference codes of each kit were k.083-2, k.016, k.082-2, k.031-2 and k.117-2, respectively. These test kits are traceable to reference material NIST (National Institute of Standards and Technology) SRM 911C, SRM 914, SRM 917, SRM 927D and SRM 1951. The biochemical tests were performed using the standard method of the company. The original concentration ranges of glucose, triglycerides, creatinine and total cholesterol were 0.781 to 100, 1.56 to 200, 0.031 to 4.0, and 1.56 to 200 mg dL^{-1}, respectively. For total protein, the original concentration range was 0.031 to 4.0 g dL^{-1}. These units are important for representation of the levels that are allowed for these compounds in clinical analysis. For example, the levels of these compounds that are 'desirable' in the blood serum or the plasma of an adult man, according to the Bioclin kit's instruction manuals, are 0.4 to 1.4 mg dL^{-1}, 65 to 99 mg dL^{-1}, less than 200 mg dL^{-1}, equal to or less than 160 mg dL^{-1} and 6.0 to 8.0 g dL^{-1} for creatinine, glucose, total cholesterol, triglycerides and total protein, respectively.

For the synthetic tests, we performed eight dilutions in water based on the initial concentration of the biochemical compound that was immobilized. The first sample was undiluted (100%). The second, third, fourth, fifth, sixth, seventh and eighth samples had 50.0%, 25.0%, 12.5%, 6.25%, 3.12%, 1.56% and 0.781% of the initial concentration, respectively. This form of dilution was applied for all the parameters that were tested.

Microcentrifuge tubes were filled with the samples, centrifuged and incubated for 10 min at 37 °C. Then, 250 μL of each biochemical sample was inserted into the wells of a 96-well ELISA microplate (available from Fisher Scientific). If the volume that is added is different from 250 μL, then the intensity of the resulting image will also be different. In that case, a new model would need

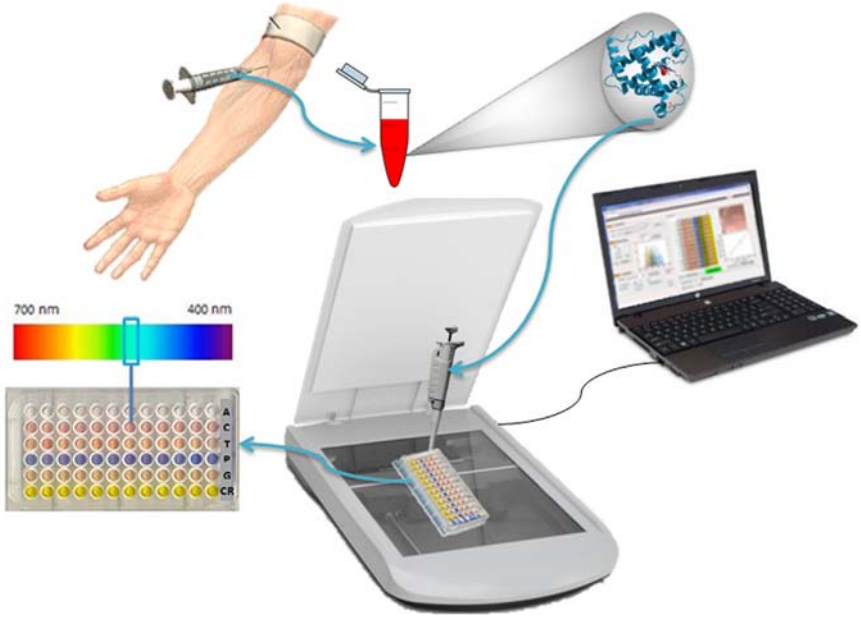

Fig. 1. Experimental arrangement to collect and analyze images to determinate the concentration of glucose, triglycerides, creatinine, total cholesterol and total protein in blood serum.

to be created, with a new calibration curve. Our models were created to read and analyze the intensity of wells with a sample volume of 250 μL.

Experiments were performed with human blood from four adult volunteers. Each of the volunteers donated 7.0 mL of blood for the analyses. The blood was placed in test tubes and centrifuged for 10 min at 2000 rpm. The blood cells descended to the bottom of the tubes, and the supernatants were collected and added to Eppendorf® tubes. Then, the serum was allowed to react with the reagents of each biochemical assay for 10 min at 37 °C. For the assay, both the scanner and the colorimetric reference method were applied on the same day. The test was performed in triplicate for each patient. Thus, three wells of the microplate were filled with different blood serum samples from the same patient. For the reference method, three flasks were prepared for each biochemical parameter, with serum provided by the same patient.

The methods utilized for colored reactions were enzymatic colorimetric methods. The cholesterol oxidase p-aminophenol (COD-PAP) method [16] was used for total cholesterol and the reaction occurs when the cholesterol esters react with the lipoprotein lipase forming cholesterol and fatty acids. The cholesterol reacts with oxygen and cholesterol oxidase, forming cholesterol-3-one and hydrogen peroxide. The hydrogen peroxide reacts with phenol, 4-aminoantipyrine and peroxidase forming water and a cherry chromogen. The cherry chromogen has an intensity of red ($\lambda = 500$ nm) proportional to the concentration of cholesterol. The Jaffe modified method was used for creatinine [17]. The creatinine reacts with picric acid, forming a reddish-yellow complex whose color ($\lambda = 510$ nm) is proportional to the concentration of creatinine. The enzymatic colorimetric glucose oxidase p-aminophenol (GOD-PAP) method was used for glucose [18]. The glucose reacts with oxygen, water and GOD, forming gluconic acid and hydrogen peroxide. The hydrogen peroxide, in the presence of peroxidase, reacts with 4-aminoantipyrine and phenol, forming a cherry chromogen whose color intensity ($\lambda = 505$ nm) is proportional to the concentration of glucose. The enzymatic colorimetric method for triglycerides [19] was as follows: the triglycerides react with water and lipoprotein lipase, forming glycerol and fatty acids. The glycerol reacts with adenosine triphosphate (ATP), glycerol kinase and magnesium(II) ions, forming glycerol-3-phosphate and adenosine diphosphate (ADP). The glycerol-3-phosphate reacts with oxygen, glycerol phosphate and oxidase dihydroxyacetone, forming phosphate and hydrogen peroxide. The hydrogen peroxide reacts with 4-aminoantipyrine, p-chlorophenol and peroxidase, forming a cherry chromogen and water. The color intensity of the cherry chromogen ($\lambda = 500$ nm) is proportional to the concentration of triglycerides. The Biuret method was used for total protein [20]. The peptidic bindings of the proteins (–CONH–) react with cupric ions, in an alkaline environment, forming a violet-colored complex whose color ($\lambda = 545$ nm) is proportional to the concentration of the total protein.

2.2. Digital image acquisition

Once the reactions were completed, we filled the ELISA microplate and performed a scan (Fig. 1). Simultaneously, analyses were carried out with the reference method. The method was performed in triplicate, and so we acquired three digital images and three values of the concentrations for each patient. Each final value was then reported as the mean of the triplicates ± standard deviation. After obtaining digital images of the microplates with the synthetic models in .BMP format, the next step was to convert the images into .TIFF format and then to extract the regions of interest. The free software GIMP 2.0 [20] was used to capture an elliptical portion of each region. In all cases, wells containing the blank (water) were also measured. The elliptical section (region of

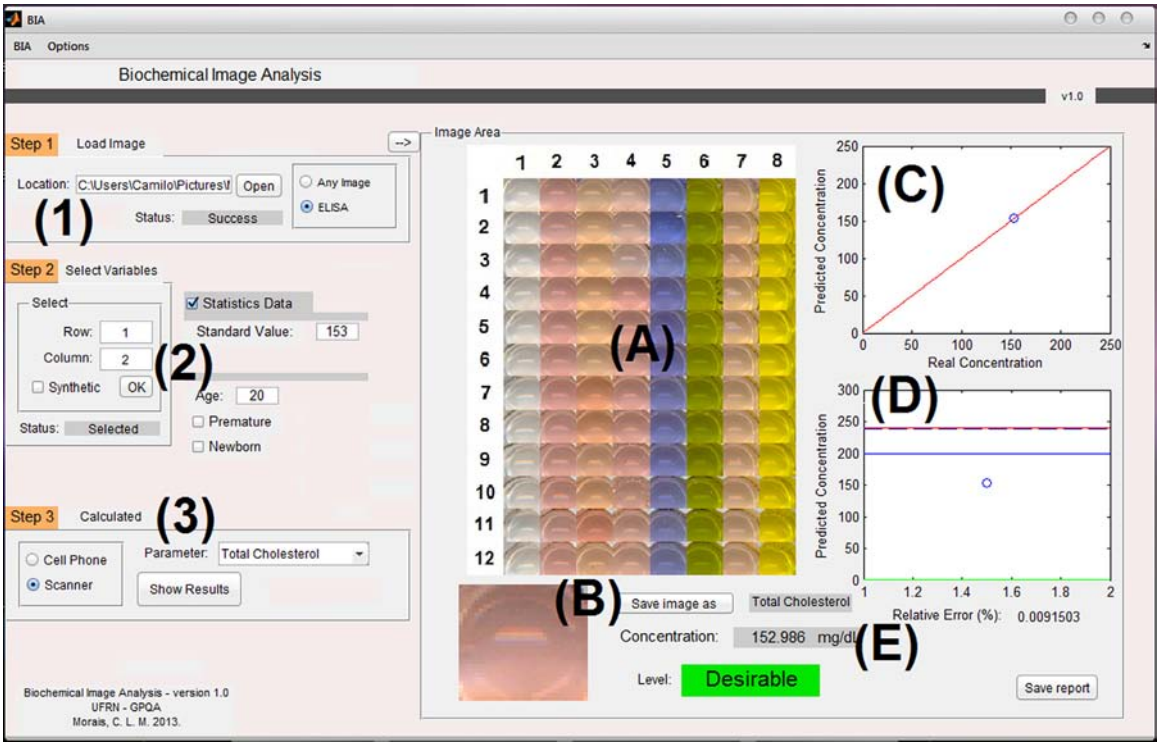

Fig. 2. Main screen of the graphical interface BIA (Biochemical Image Analysis): (1) load the image; (2) select the image and show statistical data; (3) predict the concentration of the selected biochemical parameter. (A) main view of the microwell plate with identification of the rows and columns of the wells; (B) image view of the selected well, including an option to save; (C) linear curve of the measured concentration *versus* the concentration predicted; (D) graphic with the concentration-thresholds (low, desirable, acceptable and high); (E) value of the predicted concentration with the level of health condition, the relative error and a button to save a report of the analysis.

interest, ROI) of each microwell on the microplate was chosen in such a way to obtain good signal homogeneity and symmetry, thus reducing test zone imperfections and border fluctuations. The resulting ROIs varied between 3.84 KB and 5.65 KB with a resolution of 43 × 48 pixels.

2.3. Instrumentation

The microplates were placed at the center of an HP Scanjet G2410 scanner (Hewlett-Packard, USA). The microplate is transparent, and so the digital image was acquired from the back side of

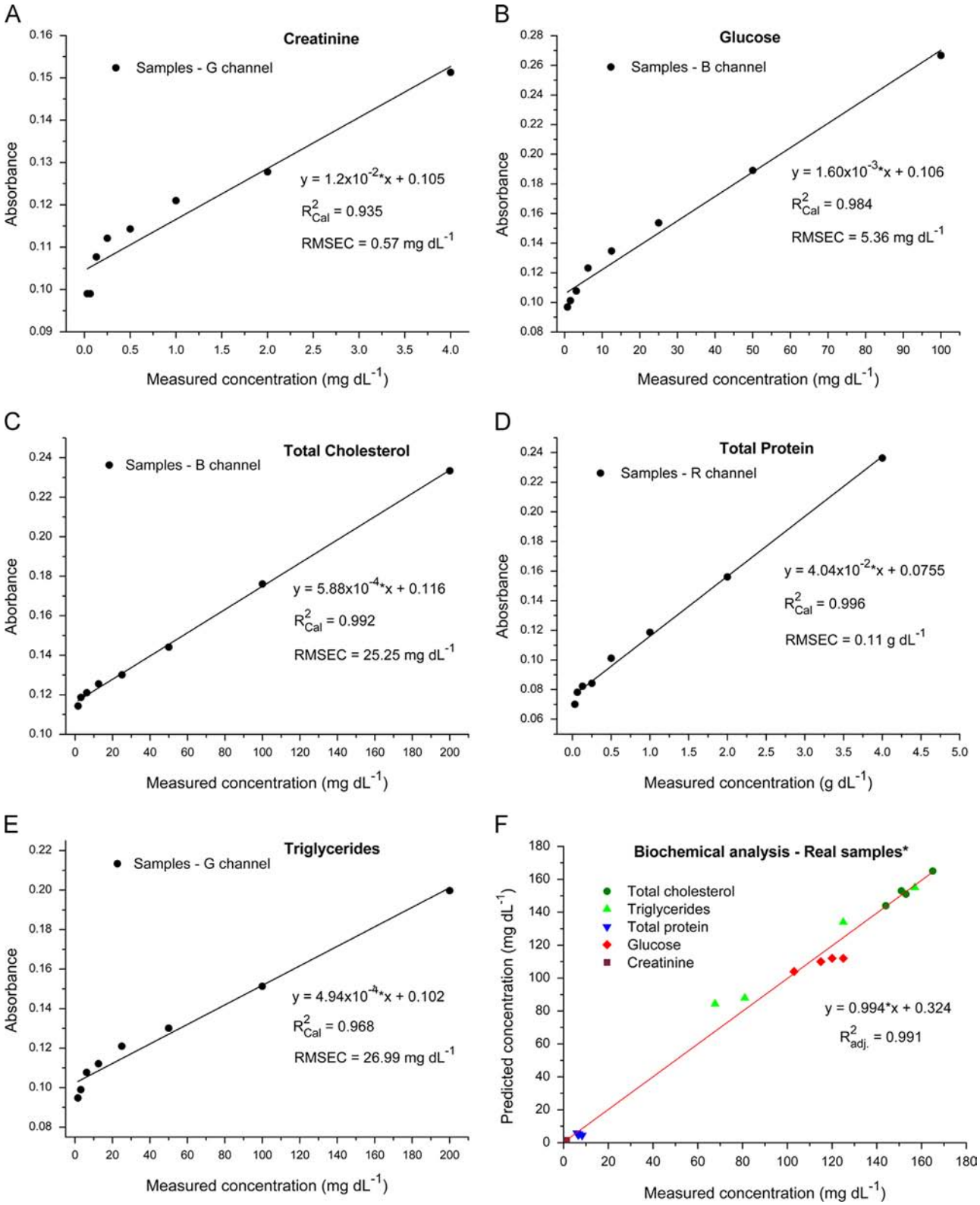

Fig. 3. Calibration and prediction curves for: A – creatinine (G channel); B – glucose (B channel); C – total cholesterol (B channel); D – total protein (R channel); E – triglycerides (G channel); F – real samples. *The concentration of total protein was divided by 1000 to adapt its concentration range in the graphic.

the plate. Each image had a resolution of 1200 × 1200 dpi and a size of 701 × 1058 pixels. For the reference method, the colorimetric equipment that was used for the biochemical analysis was the BIO-2000 (Bioplus Ltda, Brazil). This equipment had a semi-automatic calibration, where each biochemical parameter was tested to obtain the real concentration in each well from the ELISA microplate.

2.4. Data analysis

The scanned images were then loaded into MATLAB® software version 7.12 (MathWorks, USA). Each RGB digital image is formed by multiple constituent elements called pixels. The colored images consisted of three matrices, which correspond to channels R (red), G (green) and B (blue). The images were loaded into Matlab, and the RGB matrices were separated.

The next step was to calculate the intensity of the RGB channels. The intensity value was calculated as the average of the values of these matrices. Then, we rounded these values of intensity into integer values within the range of 0 to 255. The values were stored in the 'uint8' MATLAB format (8-bit elements). Next, we calculated the absorbance. For this, we used the Beer–Lambert law:

$$A = -\log \frac{I}{I_0} \quad (1)$$

where A is the absorbance; I is the intensity of the R, G and B channels; and I_0 is the maximum value of a pixel: 255. The last step for calibration was the fitting of a linear equation between absorbance and concentration. The best channel was found for each biochemical parameter that was analyzed. For total cholesterol, triglycerides, total protein, glucose and creatinine, the best channels were B (blue), G (green), R (red), B (blue) and G (green), respectively.

Real blood samples were employed for prediction of the investigated parameters. The image of each well of the plate was elliptically cropped, generating a 43 × 48 pixel image formatted by the software GIMP 2.0. Absorbance values were calculated with Matlab using the same method as in the calibration step. Knowing which color channels provided the best linear correlation for each tested parameter, the absorbance values were then converted into concentration values. The performances of the different models were evaluated by means of the Root Mean Square Error of Cross Validation (RMSECV), the Root Mean Square Error of Prediction (RMSEP) and the R^2 parameters for the data sets of the external validation samples (predicted *versus* reference values).

3. Results and discussion

Image analysis of the colorimetric reactions in the microplates produced tightly clustered red, green, and blue values for each microwell. The central values of these clusters were then transformed into absorbance values. The minimum requirement for a transparent scanner image to be an effective substitute for the traditional absorbance measurements is the ability to produce calibration curves using at least one of the red, green or blue channels for the established colorimetric reactions.

We constructed a Graphic User Interface (GUI) to automate the mathematics and statistics, thereby improving the analytical frequency of the proposed method. The interface was built in the Matlab environment, and the executable software is intended to run in Microsoft Windows® with or without the Matlab software being installed. Fig. 2 shows the BIA (Biochemical Image Analysis) GUI that was created to process an unknown image for total cholesterol assay. In the GUI, (1) is to load the image; (2) is to select the image and show statistical data; and (3) is to predict the concentration of the selected biochemical parameter. In addition, the following are provided: (A) a main view of the microwell plate with identification of the rows and columns of the wells; (B) an image view of the selected well, including an option to save; (C) if the measured concentration was shown in step 2, this graphic shows the linear curve of the measured concentration *versus* the concentration that is predicted by the model; (D) a graphic with the concentration-thresholds, indicated by Bioclin, to classify the level of concentration in the blood as low, desirable, acceptable and high for health; and (E) the value of the predicted concentration with the level of health condition, the relative error and a button to save a report of the analysis. The software does not require a highly experienced user and it is freely available and will be provided upon request *via* e-mail. Please contact the corresponding author *via* e-mail.

Fig. 3 shows the calibration and predicted curves for real samples. In some cases, such as total cholesterol, all three primary colors were correlated with analyte concentration and the line with the steepest slope was chosen as the most sensitive. In other cases, linear behavior is obtained for the absorbance signal as a function of sample concentration for a specific channel (R, G or B). Glucose, for example, was evaluated with the blue channel. The primary color that is the most sensitive for each method generally agrees with the wavelength chosen for analysis by the spectrophotometer. The correlation coefficients were 0.992, 0.984, 0.967, 0.996 and 0.998, and the RMSEP values, using the prediction set (5 samples), were 8.05 mg dL^{-1}, 7.69 mg dL^{-1}, 0.29 mg dL^{-1}, 1.41 mg dL^{-1} and 2.23 g dL^{-1} for glucose, triglycerides, creatinine, total cholesterol and total protein, respectively. These results demonstrate that good calibration curves were obtained for all parameters.

The proposed method was applied to determine glucose, triglycerides, creatinine, total protein and total cholesterol in human blood samples. Four volunteers were tested in triplicate. The results are shown in Table 1.

Table 1
Predicted concentration in real human blood samples[a].

	N[b]	Bioplus[c]	Scanner
[Glucose] (mg dL^{-1})	1	115.0 ± 2.0	110.0 ± 8.5
	2	125.0 ± 1.7	112.0 ± 2.4
	3	120.0 ± 1.5	112.0 ± 2.4
	4	103.0 ± 2.0	104.0 ± 1.2
[Triglycerides] (mg dL^{-1})	1	81.0 ± 1.0	87.9 ± 15.4
	2	67.7 ± 1.5	84.4 ± 7.8
	3	157.0 ± 4.3	155.0 ± 24.9
	4	125.0 ± 2.8	134.0 ± 27.2
[Creatinine] (mg dL^{-1})	1	1.3 ± 0.05	1.5 ± 0.4
	2	0.83 ± 0.05	0.60 ± 0.1
	3	1.4 ± 0.05	0.92 ± 0.1
	4	1.2 ± 0.05	1.2 ± 0.1
[Total cholesterol] (mg dL^{-1})	1	151.0 ± 3.0	153.0 ± 4.6
	2	153.0 ± 17.0	151.0 ± 7.1
	3	165.0 ± 8.3	165.0 ± 2.7
	4	144.0 ± 8.0	144.0 ± 7.9
[Total protein] (g dL^{-1})	1	6.0 ± 0.2	5.9 ± 0.8
	2	6.9 ± 0.3	5.2 ± 0.05
	3	6.5 ± 0.1	4.9 ± 0.04
	4	8.3 ± 0.7	4.5 ± 0.1

[a] Assays performed in triplicate for each patient, using blood serum. Each patient is represented by numbers 1 to 4. Concentration values are shown as mean of triplicates ± standard deviation.
[b] Number of sample.
[c] Reference method based on BIO-2000 equipment used for biochemical analysis.

Table 2
Figure of merits for biochemical compounds tested. The linear response, limit of detection (LOD), recovery and relative error of the image method; and the linear response and limit of detection of the reference method are shown.

Compound	Image method				Reference method	
	Linear response (mg dL^{-1})[a]	LOD (mg dL^{-1})[a]	Recovery (%)	Rel. Error (%)	Linear response (mg dL^{-1})[a]	LOD (mg dL^{-1})[a]
Total cholesterol	25.0–200	41.5	98.7–101.3	0.922	1.46–500	1.47
Creatinine	0.063–0.50	0.12	65.7–115.4	24	0.0377–10	0.0395
Glucose	12.5–125	16.2	89.6–101	6.93	1.51–500	1.51
Triglycerides	25.0–200	51.7	98.7–124.9	8.95	2.81–900	2.84
Total Protein	$0.50–4.0 \times 10^3$	6.2×10^2	54.2–98.3	32	$0.022–12 \times 10^3$	43

[a] Concentration of total protein was converted from g dL^{-1} to mg dL^{-1}.

In all cases, no statistically significant differences were observed at a confidence level of 95% between the scanner and photometer when applying the paired *t*-test. The linear response, limit of detection, recovery and relative error were calculated for the image method (Table 2). The linear response and limit of detection were compared with the reference method. The reference method is much more sensitive than the new image method, but the image method has achieved a good recovery for the predicted parameters and good limit of detection for clinical issues. The relative error in creatinine was high because its concentration in human blood is very low (0.83–1.4 mg dL^{-1}). The greatest difference between the real and the predicted concentrations was 0.48 mg dL^{-1} for this parameter. For total protein, the relative error was elevated because the test samples had their concentration range (6.0–8.3 g dL^{-1}) above the calibration curve (0.030–4.0 g dL^{-1}).

Although these results are encouraging, there were a few problems with the method, such as bubbles at the surfaces of the wells, light inside the scanned shape, altering the intensity of the images and the position of the microplate on the scanner. These physical problems can cause errors in the predicted concentrations, as can be observed with the triglycerides (sample 2) and the total protein (samples 3 and 4) in Table 1.

4. Conclusion

The results of this study show that digital image analysis could be a substitute for the spectrophotometric measurements that have traditionally been used for biochemical assays (glucose, creatinine, triglycerides, total cholesterol and total protein) of blood. The method can be used with a two-dimensional microplate array for home tests. The advantages of this method are as follows: a reduction in the amount of reagents and wastes because 250 μL of total reagent is used instead of the average 2.0 mL that is used in the standard method; a reduction in costs because the cost of a microplate is quite low (US$ 4.00) for a base unit, as is the cost of the scanner (US$ 200.00); and an increase in the analytical frequency because image analysis with the microplate has the potential to perform 96 tests at the same time. This approach can be used in remote areas and/or in non-developed countries, as an alternative method that is not expensive. In addition, this method has the potential to be made portable for analyzing results in remote locations by operating the scanner with a generator or a storage battery with an inverter.

Acknowledgments

Camilo de Lelis Medeiros de Morais thanks Propesq – PIBIC – UFRN and Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) for financial support. K.M.G. Lima acknowledges the CNPq/Capes project (Grant 070/2012) and FAPERN (Grant 005/2012) for financial support.

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