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**Quantification of Milk Adulterants (starch, H₂O₂, and NaClO) using Colorimetric
Assays coupled to Smartphone Image Analysis**

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Abstract

In this paper, a colorimetric method for the detection of milk adulterants using smartphone image analysis is reported. This is based on the reactions to detect hydrogen peroxide, sodium hypochlorite, and starch in milk, where a color variation is observed for each substance. The image analysis was performed by using lab-made apps (PhotoMetrix®, and RedGIM®) based on partial least squares regression with the histograms of the red-green-blue images. The image histograms are automatically calculated using the smartphone camera and processed within the app. The results have shown the capability of this method to predict the concentration of the three adulterants, demonstrating the potential of the use of digital images and smartphone applications associated with chemometric tools. This method presents a fast, low-cost, and portable way to quantify adulterants in Cow milk.

Keywords: Milk; adulterants; RGB image; PLS; smartphone;

1. Introduction

Cow milk is one of the most consumed foods throughout the world. It has a high nutritional value, providing essential nutrients such as water, carbohydrates, fat, proteins, minerals, and vitamins.[1] However, milk can be easily adulterated. For example, the addition of water, [2] starch, hydrogen peroxide (H_2O_2),[3] and sodium hypochlorite ($NaClO$) [4] are often observed. Starch is added with the aim to mask extra addition of water, increasing its solid-not-fat (SNF) content.[3,5] Hydrogen peroxide and $NaClO$ are added to the milk as preservatives to inhibit or delay the appearance of microorganisms and to prolong the product shelf-life.[3,5] However, these compounds are harmful to humans. Excessive ingestion of starch can cause diarrhea due to undigested effects in the colon, and accumulation of starch in the body may be fatal for diabetic patients. Preservatives such as H_2O_2 and $NaClO$ can cause gastritis and inflammation of the intestine due to gastrointestinal complications.[5]

There are several methods for detecting milk adulterations.[6,7,8,9,10] Starch can be determined by using many approaches, such as iodine titration with potentiometric or amperometric detection,[11] near-infrared spectroscopy, [12] and a complexation reaction of iodine with starch, which the last has been used as a proper methodology in Brazil.[3] Detections of peroxides in milk, i.e., H_2O_2 and Na_2O_2 , can be performed by many methodologies, generally using colorimetric or electrochemical methods [3,13,14] as well as from Rhodamine B reagent [15] or neutralization reactions.[4]

Continuous monitoring of milk is a serious issue, and alternative methodologies that can reduce cost, automatize, and speed up this type of analysis are very significant. The use of digital images as a detection tool of milk adulteration is an excellent example of this. Some papers have reported methodologies for the detection of water, caustic soda, H_2O_2 , synthetic urine, and synthetic milk in milk samples.[2,16] This tendency occurs because, nowadays, devices that capture and process digital images are in our pockets. The use of smartphones as

analytical devices has been reported in many applications, including clinical, [17,18] forensic, [19,20] food, [21] and environmental [22] analysis. Recently, it was developed in Brazil, a smartphone application (PhotoMetrix®) that allows the performing image acquisition and treatment of data obtained in the device itself. With this, it is possible to prepare, based on the RGB system, the calibration and determination of the concentration of an analyte of interest in the sample. This application has been used in several studies such as quantification of iron in vitamin supplements and banknote differentiation, [23] indirect iodine analysis in biodiesel, [24] thermal stability of milk, [25] quantification of ethanol in cachaça, [26] identification of tannins, [27] document authenticity, [28] and monitoring of fluorine in alternative water supply systems [29].

The digital images are usually based on the red-green-blue (RGB) color system, where each color channel has an 8-bit scale. Therefore, each pixel can assume one of 28 possibilities of intensity values (0–255).[30] The mix between these three colors generates all other colors, which can be perceived by a visible spectrum region. Color histograms are often used as a source of information on RGB images. This histogram describes the statistical distribution of the pixels as a function of the color component. [31] An essential aspect of color histograms is that it can be used as input data for the multivariate analysis since it has a one-dimensional data structure similar to a spectrum.

In this paper, an alternative method for the detecting of adulterated cow milk by starch, H₂O₂, and NaClO is described based on RGB images acquired with a smartphone. All data processing was performed using two apps, PhotoMetrix® (www.photometrix.com.br), and RedGIM® [32] within the device, enabling the realtime analysis of milk samples.

2. Experimental procedure

Materials and Reagents

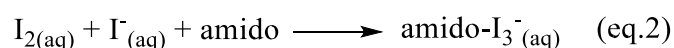
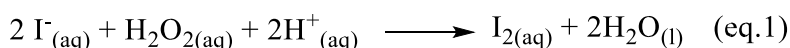
Ultrapure water (resistivity = $18.2 \text{ M}\Omega\cdot\text{cm}$) produced from the osmosis system (Purelab Ultra Mk2, United Kingdom) was used to prepare the solutions. Starch (Sinhá – Maringá, Paraná, Brazil) and 10% iodopolyvidone solution (1% active iodine – Rioquímica – São José do Rio Preto, São Paulo, Brazil) were used for the identification of starch. For the detection of H_2O_2 , solutions of hydrochloric acid (HCl) 1% v/v (Dynamica - Indaiatuba, São Paulo, Brazil), potassium iodide (KI) 10% w/v (CRQDiadema, São Paulo, Brazil) and starch 1% w/v were used. Potassium iodide solutions 7.5% w/v and starch were employed for detection of NaClO . A warming blanket (Fisatom 22E – Perdizes, São Paulo, Brazil) and vortex agitator (Vixar – Jacareí, São Paulo, Brazil) were also used.

Samples

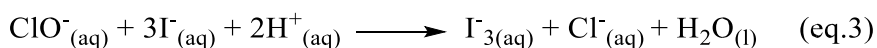
A sample of natural milk from a producer located in Vargem Alta city, Espírito Santo state (Brazil), was collected and sent to the Petroleomic and Forensic laboratory. This sample was considered unadulterated and used for the construction of the analytical curve. The sample was kept in a polyethylene bottle and chilled between 6 and 10 °C. Seven brands of milk (Ibituruna®, Selita®, Parmalat®, Milky®, Italac®, Cotochés®, and Piracanjuba®) were acquired in supermarkets in the metropolitan region of Grande Vitória to be analyzed. Each brand of whole milk was identified numerically (1-7) and analyzed according to the analytical standards of the Instituto Adolfo Lutz. [33] For each adulterant, a specific test was performed (Figure 1) through iodine reactions indicating the presence of the adulterant by the appearance of specific staining, being quantified by the capture of the image in the region of interest from the use of apps.

Colorimetric assay and analytical curve construction

The analytical curve was constructed from known concentrations. For each adulterant, an analytical curve was constructed in test tubes. The construction of the analytical curve for the H₂O₂ adulterant (0 – 1.5% v/v) was performed utilizing a commercial solution of H₂O₂ 3%. In a test tube, each point of the analytical curve was added a specific volume of this solution considering a final volume containing neat milk and adulterant equal to 2 mL. To this solution was added 2 mL of 1% v/v HCl and 2 mL of 10% w/v KI solution. To confirm the presence of H₂O₂, it was added 1% w/v of starch occurring, then, the development of the blue coloration (as explained from **equations 1 and 2**).



For the NaClO curve (0 – 10 %v/v), specific volumes were added in test tubes from a concentrated solution for each point of the curve considering the final volume equal to 5 mL (neat milk plus adulterant). Then, 0.5 mL of 7.5% w/v KI solution was added, and the solution was stirred. The development of the yellow staining indicates the presence of free chlorine. For confirmation, 1 mL of 1% w/v starch solution was added, indicating the presence of NaClO by the development of blue staining (**equations 2 and 3**).



For starch adulterant (0 – 15 %v/v), the curve was prepared from an aqueous starch solution (25 mg mL⁻¹), and for each point was added a specific volume (50-3.000 µL) of adulterant *in natural* milk considering final volume equal to 5 mL. Then, it was added two drops of 1% polyvidone iodine solution. The development of blue staining indicates the presence of starch in the solution (**equation 2**).

To evaluate the accuracy of the developed method, the addition of controlled concentration of adulterations was performed in the samples of commercial milk from an aqueous solution of each adulterant being starch (A), hydrogen peroxide (H₂O₂) and sodium hypochlorite (NaClO).

Root Mean Square Error of Cross-Validation (RMSECV – **equation 4**) was used to evaluate the prediction accuracy of the method.

$$RMSECV = \sqrt{\frac{\sum (yp - yc)^2}{n}} \quad (\text{eq. 4})$$

Where: yp = predicted concentration value; yc = expected value; n = number of calibration samples.

Figure 1: Flowchart of the methodology used to quantify the adulterants in milk via PhotoMetrix® and RedGIM® apps.

Data analysis

Data analysis was performed through the PhotoMetrix® [34], developed for Android, IOS and Windows phone systems, and RedGIM® applications [32] (which works only for Android and Windows phone systems). In these applications, the images are acquired using the smartphone's camera, which, in this case, it was a Samsung S7 smartphone with 12-megapixel camera resolution. Subsequently, a determined region of interest (ROI), with the size of 32x32 (for PhotoMetrix) and 50x50 (for RedGIM) for each image sample, it is collected and used as input data for calculating the histogram. The RGB color histogram is calculated for each color channel separately. The resulting histogram is normalized by a blank image to

correct the ambient variations on the images. Finally, all histograms are organized into a calibration and prediction data set for use in a partial least squares regression (PLS) calibration technique.[25]

3. Results and discussion

The addition of different substances into the natural composition of the milk is directly linked to fraud and adulteration processes, which can occur from milk harvesting to the marketing phase. It is prohibited any conservative, neutralizer, density constituent in the chemical composition of milk, and if they are detected, milk or dairy products may be used only for casein or soap production. [35]

The presence or absence of adulterant was evaluated employing colorimetric tests already developed and standardized.[25] Typically, these assays are only a qualitative analysis, but herein, a new analytical technique for quantification of adulterants was developed to improve the quality control of this food. The use of the smartphone application is a new proposal to be used, thus indicating the amount in the concentration of each adulterant since commercial milk samples of poor quality can cause serious health problems.[36,37]

For the development of the quantification method using the PhotoMetrix® and RedGIM® apps, an analytical curve was constructed varying the concentration of the adulterants from 0-1.5% v/v, 0-10% v/v and 0-15% w/v for the H₂O₂, NaClO and starch analytes, respectively. The results of the colorimetric assays are shown in Figure 2a, c, and e, which they present variation in color intensity due to the analyte concentration present in the solution. Therefore, the higher intensity of the color in the solution refers to the higher concentration of the analyte of interest. Using the multivariate calibration associated with the

digital image for the PhotoMetrix® app, we obtained the results of linear regression with a coefficient of determination to calibration (R^2) always greater than 0.9929, as shown in Figure 2b,d,f. The values of R^2 , RMSEC, RMSECV and calibration curve equation obtained for each adulterant are described in Table 1. The lowest values of RMSECV were observed for the detection of the H₂O₂ adulterant (0,313% v/v, 0,083% v/v respectively). The RMSECV value is used to evaluate the accuracy of the sample prediction. Conversely, the lowest analytical sensitivity was observed for the quantification of the NaClO (with values of RMSEC = 0.5087% v/v, and RMSECV = 5,46% v/v), Table 1.

A similar multivariate calibration model by PLS was also developed for the RedGIM® app, and the results are shown in Figure 3 and Table 1, where the R^2 values were always higher than 0.9653 with RMSEC from 0.0104 to 1.100. It should be noted that the Redgim application does not present the possibility of to export the RMSECV data, so they were not reported in this work. Generally, the analytical performance observed between the two applications was similar and reproducible.

Figure 2: Analytical curve constructed for quantification of starch (a), NaClO (b), and H₂O₂ (c) in milk and their respective results of PLS (b, d, and f) using the PhotoMetrix® app.

Figure 3: Analytical curve constructed for quantification of starch (a), NaClO (b), and H₂O₂ (c) in milk and their linear regressions (g, h, and i) for the RedGIM® app.

Table 1: Figures of merit for the determination of adulterants in milk by using PhotoMetrix® and RedGIM® apps.

From the built PLS model, the concentration of H₂O₂ present in seven commercial milk samples was quantified. The samples were identified in numerical order of 1-7. The analyses were performed in duplicate, and the accuracy of the method was checked with the addition of a known H₂O₂ concentration (0.3% v/v), thus verifying the reproducibility and reliability of the analytical data.

Visually, it can be observed that all samples presented negative results for H₂O₂ since none presented alteration of color from white to yellow (Figure 4a), characteristic staining due to the reaction of the KI with the H₂O₂ having as the product the formation of potassium hydroxide and iodine, which confers yellow coloration to the solution. Thus, the higher the yellowish hue of the sample, the higher the amount of H₂O₂ present in the milk.

Figure 4: Commercial milk samples and samples with addition of (a) 0.3 % v/v H₂O₂, (b) 4 % v/v NaClO and (c) 3% m/v starch.

The PhotoMetrix® app reports the data in predicted values based on the analytical curve and according to the number of latent variables. For this analysis of PLS, three latent variables were used, obtaining 58.5% of the dependent values for 99.97% of the predicted values. For the samples commercialized (Table 2), it is verified that the mean values of predicted H₂O₂ are lower, ranging from 0.01 to 0.06% v/v.

For the samples of the addition of known concentration of H₂O₂, 0.3% v/v, the values detected ranged from 0.27 to 0.47 % v/v, showing the efficiency of the colorimetric test associated with the PhotoMetrix® app in the detection and quantification of H₂O₂. Comparing the results obtained of Table 2 with the RedGIM® app (Table 1S), it can be observed that all commercial samples presented lower concentrations, ranging from 0.259 to 0.27% v/v. For the samples with the addition of H₂O₂, the values ranged from 0.202 to 0.395% v/v.

Table 2: Quantification of H₂O₂ in commercialized milk samples using the PhotoMetrix® app.

The presence of NaClO traces may appear in milk due to its use as sanitizers and by the cleaning process of equipment used for the milk production. However, the substance may have been deliberately added to increase the shelf time of the product.[4] For this adulterant, three latent variables were considered, where 63.43% were for dependent values and 99.29% of the predicted values. Similar to the result for the adulterant H₂O₂, all seven samples commercialized also presented negative results for NaClO, since none presented alteration of their original coloring (from white to yellow) as indicated by the assays shown in Figure 4b. The color change is provided by the iodine release from the reaction between potassium iodide and hypochlorite, forming potassium chloride and iodine. For the commercial samples, NaClO values are between 0.02 and 1.12 %v/v (Table 3).

For the assays with the addition of internal standard, i.e. [NaClO] = 4% v/v, the values determined were between 4.08 and 5.58% v/v, despite the high RMSECV values determined. Conversely, an unsatisfactory performance was obtained from the results using the RedGIM® app, Table 2S. This is due to a problem in the accuracy of the method since the known concentration samples of NaClO presented discrepant values in relation to the expected (i.e., between [NaClO] = 1.37 and 7.11 % v/v). This may be explained by the fact that this app presents a limitation in the data acquisition process, where, in order to construct the calibration curve by the PLS method as well as for its subsequent application, it is always necessary to acquire a single image, containing all the samples used in the study (calibration and prediction set). Therefore, this behavior can contribute to decreasing in the resolution or the quality of the results obtained. Considering also the instability of the chemical reaction involved for this test, due to the rapid precipitation of the compound produced, as well as the variation of the time

for each reaction, the accuracy of the method may be compromised. In contrast, in the PhotoMetrix® app, capturing the image can be done immediately after each reaction and in an isolated manner.

Table 3: Quantification of NaClO in commercialized milk samples using the PhotoMetrix® app.

For the determination of the starch concentration, it is necessary to warm the sample to facilitate the opening of the helical chain of the polymer, and then, the iodine is absorbed by the β -amylose chain. The analytical curve for this adulterant ranged from 0 to 15 % w/v (Figure 2c), and the three latent variables used provided 46.12 % of dependent values and 99.74 % of predicted values. Visually, there was no color change observed between the seven samples studied. Through the obtained PLS results (Table 4), it can be observed that for commercial samples, concentration values between 0.78 and 1.64 % w/v were determined. Therefore, it cannot be confirmed that there was adulteration for starch since the method does not present precision for quantification, requiring another analysis aiming for a more accurate result. Similar behavior was observed for the RedGIM® app, Table 3S. This fact does not indicate application inefficiency for starch quantification but that the abrupt variation in bluish coloration can cause an "overflow" in the RGB system's color intensity resulting in less accurate results. Gondim and collaborators reported that 25% of the samples analyzed presented inconclusive attributions for starch when the soft independent modeling of class analogy (SIMCA) calibration method of data obtained by FTIR spectroscopy in the middle region was used. The authors also showed that even with this inconclusive attribution, the developed analytical method worked correctly.

Table 4: Quantification of starch in commercialized milk samples by the PhotoMetrix® app.

4. Conclusion

The results obtained from the image processing by the apps demonstrated a mean coefficient of correlation of 0.9997 for H₂O₂, 0.9929 for NaClO, and 0.9974 for starch by using PhotoMetrix® and 0.9785, 0.9653 and 0.9777 for RedGIM®, respectively. Results obtained were satisfactory and can conclude that the application of PhotoMetrix® and RedGIM® apps allow the identification and quantification of adulterants in milk for the three adulterants studied, being a simple, low cost, fast, and robust analytical tool, having a high potential for food quality control. It is noteworthy that the applications still allow the creation of a database and presents a quick and straightforward form to the export of the data, which minimizes errors and increases the accessibility of the treatment of digital images.

Author Statment

Rayana A. Costa – Formal analysis, and draft of manuscript, and Writing – review & editing

Camilo L. M. Morais – interpretation data, and Software development

Thalles R. Rosa - Software development

Paulo R. Filgueiras - interpretation data

Monike S. Mendonça - formal analysis

Isabelly E. S. Pereira - formal analysis

Bruno V. Vittorazzi - formal analysis, and methodology

Marisa B. Lyra - draft of manuscript

Kássio M. G. Lima - Software development

Wanderson Romão - Resources ,and writing – review & editing

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Declaration of interests

☒ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Figures

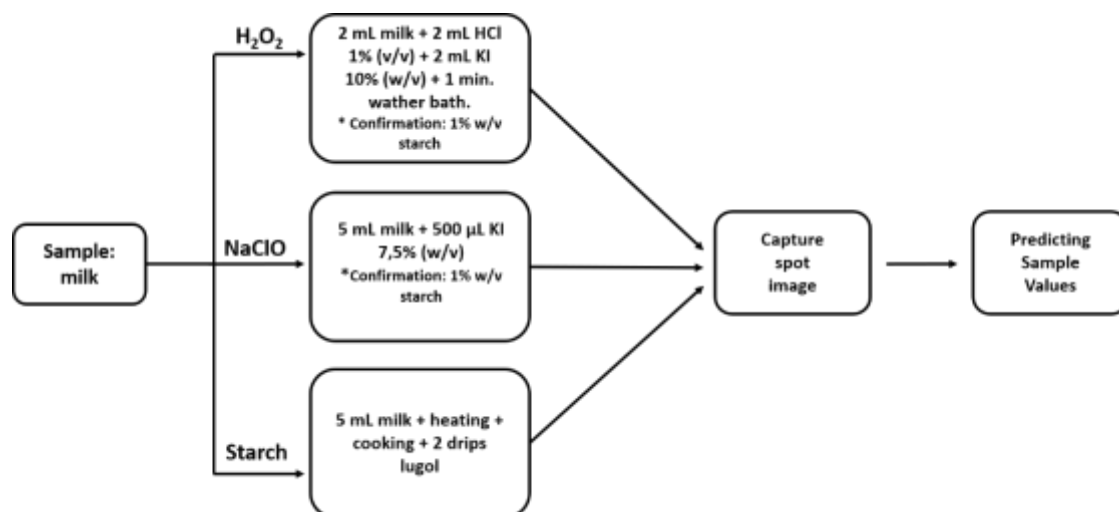


Figure 1. (print in white and black form)

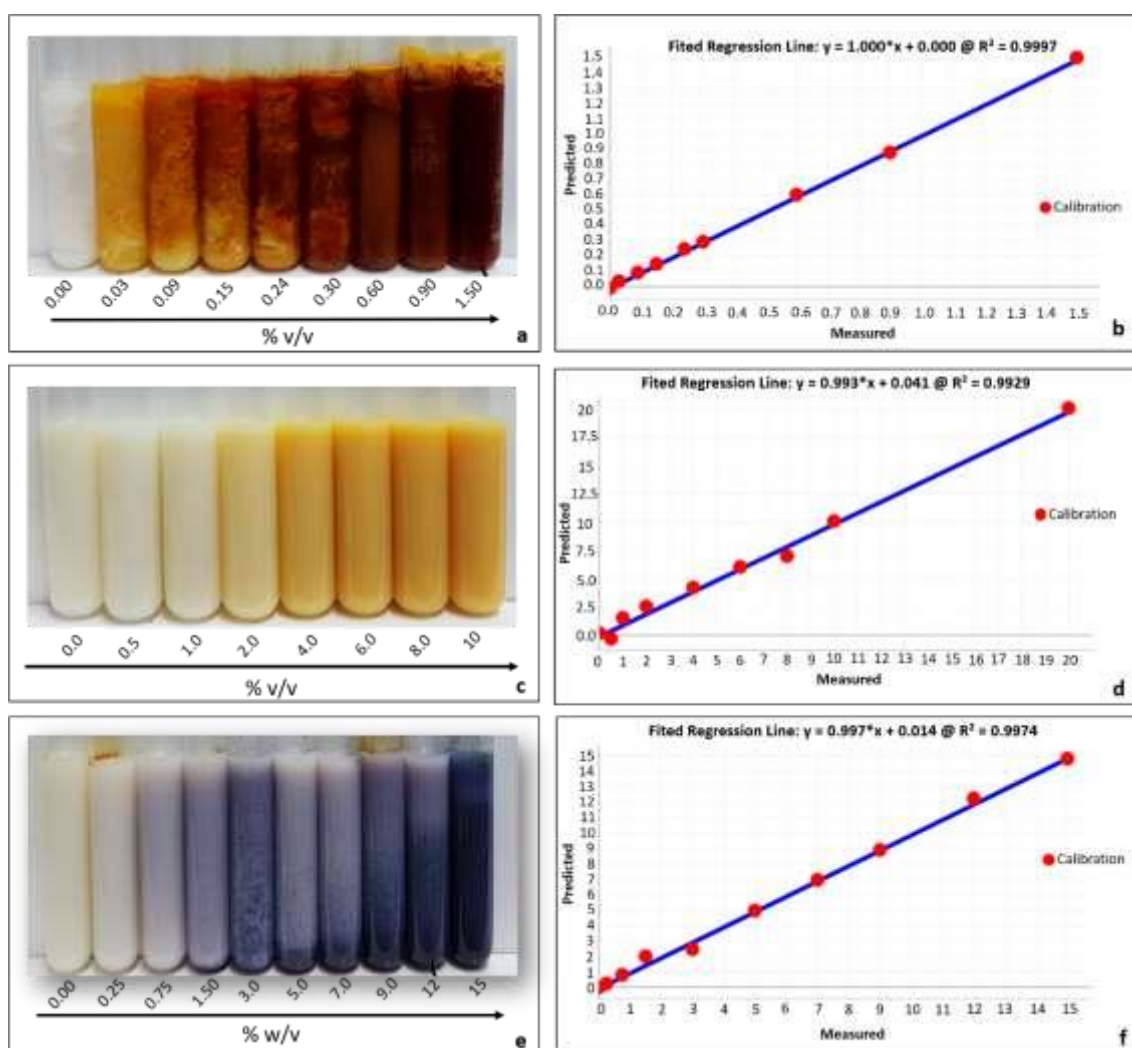


Figure 2. (print in white and black form)

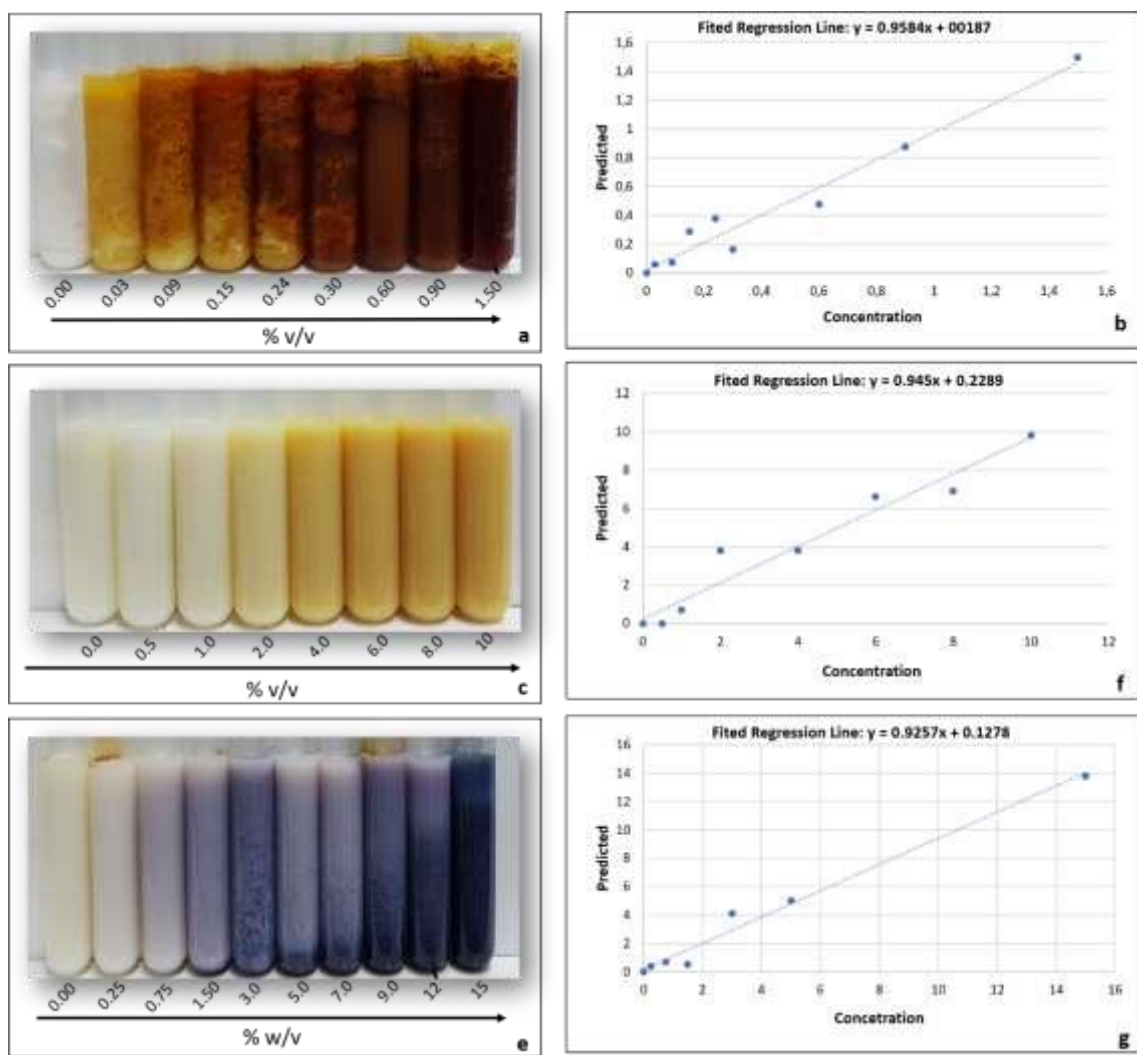


Figure 3. (print in white and black form)



Figure 4. (print in white and black form)