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A Novel Method to Assay Aspirin in Pharmaceutical Formulations by Smartphone Camera-Based Image Scanning Densitometry

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Abstract: Aspirin, a widely-used anti-inflammatory drug, can lead to serious consequences when overdosed. Therefore, there's a need for simple, cost-effective methods to determine its concentration and mitigate potential risks. This study aimed to develop a method for assessing aspirin in pharmaceutical preparations without the need for expensive equipment and with minimal sensitivity to ambient light. In this work, aspirin was subjected to a reaction with Fe(III), leading to the formation of violet-colored spots on filter paper and a 96-microwell plate. These colored spots were then captured using a smartphone in normal lighting conditions and analyzed on a computer. The integrated density of each spot was measured using a novel grayscale technique, and a calibration curve was created to relate integrated density to aspirin concentration. Analytical parameters and reagent concentrations were optimized for accuracy. To validate the method, three commercial aspirin samples were assayed and compared to ultraviolet-visible spectrophotometry, a reference method. The developed technique demonstrated excellent precision (coefficient of variation <0.68%) and relative errors below 5.2%. When compared to traditional color models like red-green-blue (RGB) and hue-saturation-luminosity (HSL), the grayscale model showed superior correlation (R^2> 0.996), while the RGB model yielded less precise results (R^2= 0.792). This study showcased the effectiveness of a cost-effective methodology for accurate aspirin quantification using a smartphone camera, even in the presence of ambient light.

Keywords: Image scanning densitometry, spectrophotometry, spot tests, smartphone, aspirin, drug analysis.

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1. INTRODUCTION

Aspirin or acetylsalicylic acid is one of the most commonly used drugs in the world and its wider usage can be estimated by the fact that over 40,000 tons of the drug are consumed each year globally (1) with more than 17 million prescriptions in the United States alone (2). Aspirin was first introduced in the market by the Bayer Company in Germany in 1899. It is generally believed that Felix Hoffmann was the first to prepare acetyl ester of the acid and developed aspirin to help his rheumatic father (3). Aspirin belongs to a group of non-steroidal anti-inflammatory drugs that are characterized by their anti-inflammatory, analgesic, and antipyretic functions. Aspirin is routinely used in a wide range of painful conditions, including head, body, and muscle aches, arthritis, and many other common ailments (4). Today, aspirin is also taken by millions of people who benefit from its antithrombotic effect (5). The medical community has increasingly recommended routine therapy with low-dose aspirin, which significantly reduces the risk of death from a cardiovascular event (6).

The pharmaceutical activity of a drug depends upon the chemical characteristics of its molecules, thus any small variation in chemical properties and quantitative composition may lead to considerable variation in therapeutic effects. Aspirin overdose has potentially serious consequences leading to many side effects including nausea and vomiting, abdominal pain, lethargy, ringing in the ears, and dizziness (7). The most common cause of death

following an aspirin overdose is cardiopulmonary arrest usually due to pulmonary edema (8). Therefore, a proper assay and quantification technique to ascertain aspirin content in a multi-component pharmaceutical dosage and its stability is indeed necessary, vital, and appreciated to avoid overdose.

Numerous classical and instrumental methods have been used for the determination of aspirin in pharmaceutical formulations including titrimetric (9), potentiometric (10), ultraviolet-visible spectrophotometry (11), ion-selective electrode (12), high-performance liquid chromatography (13), and gas chromatography-mass spectrometry (14). Titrimetric, spectroscopic, and hyphenated chromatographic methods for the analysis of aspirin from various analgesic formulations have been recently compared by Anthony et al. (15). Aspirin has been successfully determined by oxidation-reduction reaction with $KMnO_4$ (16). The most commonly used methods for the quantitative analysis of aspirin are spectrophotometric in which Fe(III) salts are used as coloring reagents to form a violet complex, the absorbance of which is used as a measure of aspirin quantitatively (17). In 2006, Kohl et al. showed that similar absorbance work could easily be performed by using colored solutions and digital images obtained with charge-coupled devices (18). Since then, there have been several reports in which smartphones, scanners, and digital cameras were used as photometers (19-21). Our group has also quantified several species, including Fe, Ni, Hg, As, formaldehyde, and sulfide at the micro level by using spot tests and a flatbed scanner (22-25).

In the present age of information technology, the number of smartphone users has increased to almost 2.87 billion. Smartphones are replacing digital cameras and scanners due to more features including high-resolution cameras, better image acquisition technology, powerful processing units, fast and easy analysis, multiple image formats, and portability, making smartphone-based colorimetric analysis ideal for research in biomedical and pharmaceutical fields (26). Recently, Soares and Rocha employed spot tests and a smartphone for the analysis of uric acid in saliva (27). Franco et al. determined Cu^{+2} in distilled beverages by fabricating a smartphone case to minimize the effect of ambient light (28). Analysis of $KMnO_4$, $CoSO_4$, $NiSO_4$, $CuSO_4$, etc. has also been carried out using smartphone camera-based techniques (29). Additionally, smartphones coupled with paper-based analytical devices (PADs) have been gaining popularity for the analysis of biomolecules and ions (30, 31). Quantitative analysis of many antibiotics like vancomycin, gentamicin, neomycin sulfate resazurin, etc. has also been carried out using smartphone-based colorimetric techniques (32-34). However, it is reported that the images captured from a smartphone suffer from the effects of ambient light and may lead to poor accuracy (35).

In literature, the most commonly used method for making colorimetric measurements is reported to be the red-green-blue (RGB), which is a subtractive color model consisting of 8-bit pixels from 0 to 255 with 0 being black, 255 pure white, and the rest of the colors fall in between the range (22). The Hue-saturation-luminosity (HSL) color space is represented in three dimensions where hue corresponds to different color values from 0° to 360° on the color wheel, while saturation is the trueness of the color. Luminosity gives lightness or darkness to a color shade regardless of its saturation (36). Recently, da Silva and Borges used a 96-microwell plate and a flatbed scanner for the quantitative analysis of aspirin in pharmaceutical tablets using the RGB model (37). They employed a complexation reaction of aspirin with Fe(III) to get violet-colored solutions in a 96-microwell plate, the RGB intensity of which was measured by using the green component in ImageJ. However, it was reported that employing the same model using a smartphone camera led to poor results (R^2 <0.77) due to the effects of ambient light. Moreover, the figure for percent errors climbed to 14.1% even using a flatbed scanner.

In the present work, we addressed this gap by employing a novel grayscale quantification technique using smartphone camera-based analysis of aspirin spot tests and 96-microwell plate solutions. The main objective of this work is to develop a technique that can be applied to the quantitative analysis of aspirin regardless of the effects of ambient light. However, the effects of ambient light on RGB or grayscale measurements are not discussed in this paper and require further research. Our proposed grayscale method is robust, simple, accurate, adequately sensitive, and can be applied to black precipitation reactions and turbid solutions as well.

2. EXPERIMENTAL SECTION

2.1. Apparatus and Reagents

Micropipette (Pipettman 20-100 µL and Pipettman 0.2-2 µL), Whatman filter paper No. 42, and disposable 96-microwell plate (CITOTEST®) were used for carrying out spot tests and developing colored solutions in microwells. Single beam visible range spectrophotometer-Jenway 6300 with wavelength range 320-950 was used for reference measurements. Sigma-Aldrich Iron(III) chloride hexahydrate ($FeCl_3.6H_2O$) was used as a complexing agent. Sodium hydroxide (NaOH) and hydrochloric acid (HCl) were purchased from Merck and deionized water was used to prepare the solutions. Pure aspirin was obtained from a local pharmaceutical firm: LAHORE CHEMICAL & PHARMACEUTICAL WORKS (PVT) LTD. Three pharmaceutically formulated drug samples of aspirin from different manufacturers (Table 1) were obtained for method verification.

Table 1: Pharmaceutical samples of aspirin used in the study.

Trade name	Contents	Manufacturer
Disprin	Aspirin 300 mg	Reckitt Benckiser Pakistan Ltd.
Loprin	Aspirin 150 mg	Highnoon Laboratories Ltd.
Ascard	Aspirin 150 mg	Acto Laboratories Ltd.

2.2. Equipment and Software

Apple iPhone X with a 12-megapixel camera was used for image acquisition of the spots with a built-in camera app and default lighting mode. Adobe Photoshop 2020 (version 21.2.2) (Adobe Inc.) was used for the quantification of spots in a desktop computer (DELL OptiPlex 3020 core i7) running on Windows 10 with additional AMD Radeon graphics (R7 200 series). Microsoft Excel (Microsoft Inc.) and OriginPro 2018 (64-bit) SR1 (OriginLab) were used for data analysis and graph plots.

2.3. Preparation of Reagents and Standards

5000 µg/mL stock solution of aspirin was prepared by dissolving 0.5 g of pure aspirin in 5 mL of 1 M NaOH. The solution was hydrolyzed by heating and stirring for 30 minutes. After heating, the solution was transferred to a 100 mL volumetric flask and made up to mark with deionized water. Using a micropipette, 20, 40, 60, 80, 100, 120, and 140 µL of aspirin stock were added in test tubes followed by the addition of 0.5% acidified $FeCl_3.6H_2O$, to prepare standard solutions of concentration: 500, 1000, 1500, 2000, 2500, 3000 and 3500 µg/mL. For the 96-microwell plate method, 1000 µg/mL aspirin stock solution was prepared by dissolving 0.1 g of pure aspirin in 5 mL of 1 M NaOH. The solution was hydrolyzed by heating and stirring for 30 minutes and afterward, transferred to a 100 mL volumetric flask and made up to mark with deionized water. 0.5% acidified $FeCl_3.6H_2O$ was prepared by adding 2.50 g of the compound in a 500 mL volumetric flask. The solution was then made up to the mark with 0.2 M HCl. NaOH and HCl were prepared in concentrations from 0.2 M to 1 M to observe the effects on color development.

2.4. Preparation of Samples

Each aspirin commercial tablet was weighed corresponding to 0.3 g and ground to a fine powder. The samples were hydrolyzed in 5 mL of 1 M NaOH with constant stirring and heating for 30 minutes on a hot plate and transferred to 50 mL volumetric flasks followed by the addition of deionized water. Working standards were prepared by necessary dilutions of the stock and made up to the mark with 0.5% acidified $FeCl_3.6H_2O$ to get violet colored solution of aspirin for both filter paper spot tests and 96-microwell plate method.

2.5. Development of Colored Spots and Image Acquisition

Three strips of Whatman filter paper were cut to approximately 14.5×1.5 cm in length. Using a micropipette, 1 µL drops of aspirin standards and samples were transferred from test tubes to filter paper. Violet-colored spots appeared on filter paper that remained stable for 5 minutes. For the 96-microwell method, aliquots of 10, 20, 30, 40, 50, 60, and 70 µL of aspirin stock were added in the first row of the 96-microwell plate followed by the addition of $FeCl_3.6H_2O$ to get violet-colored solutions with concentrations of 50, 100, 150, 200, 250 and 300 µg/mL. The solutions in each well were mixed well with the help of a micropipette tip. A similar method was used for samples followed by serial dilutions. The image of filter paper spots and 96-microwell plate was captured using iPhone X in standard lighting conditions and autofocus to avoid blurring.

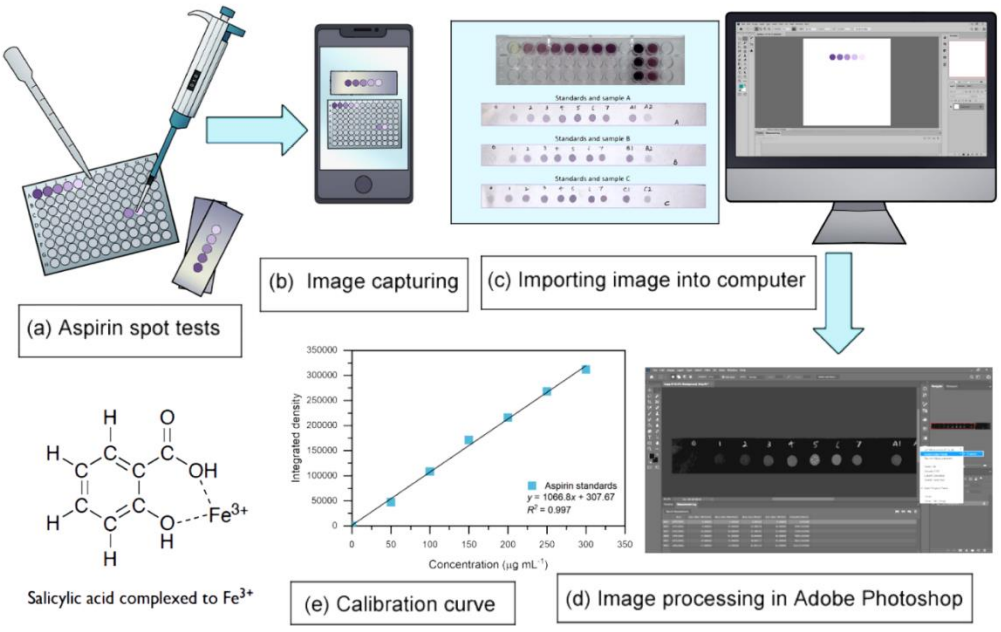

Figure 1: Graphical abstract of the proposed grayscale method for the smartphone camera-based quantitative analysis of aspirin.

2.6. Data Analysis

Images of filter paper spots and a 96-microwell plate were transferred to a computer using a USB cable in JPEG (Joint Photographic Experts Group) format. For the quantification procedure, images were imported into Adobe Photoshop in RGB mode with 8 bits per channel and converted from RGB to grayscale mode by going into the menu: Image/Mode/Grayscale. To make sure the boundaries of spots are well seen and sharp, the image was inverted in colors by going into the top menu: Image/Adjustments/Invert. The color balance of black and white was adjusted using the level correction feature as illustrated in Figure 2. The levels were corrected in such a way that the background of the image turns into pure black to give a value of 0, which minimizes the integrated density of the background and blank from the readings of standards and samples.

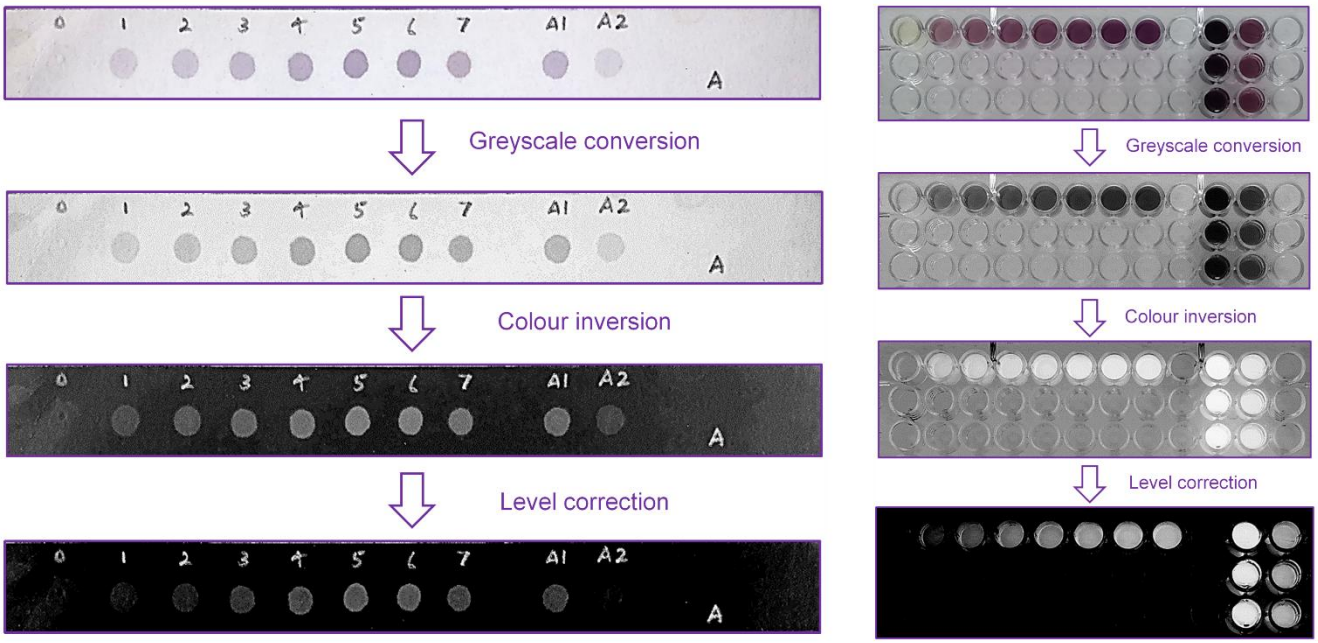

Figure 2: Image manipulation and editing of aspirin filter paper spots and 96-microwell plate in Adobe Photoshop.

After image editing, the procedure for measuring grayscale intensity was carried out using the measurement log feature in Adobe Photoshop (Figure 3). Custom data points were selected from options to get the desired values of gray maximum, minimum, mean, median, area, and integrated density. To get the values for integrated density, a selection of 50×50 pixels was made on the first spot using the elliptical marquee tool. The selection was saved by going into the top menu: select/save selection menu. The same selection was moved to the next spot by pressing CTRL+Shift and moving the cursor to the rest of the spots and selections were saved one by one. The measurements for each selection were recorded by clicking on the selection and selecting record measurements in the measurement log window. This gave values of gray maximum, minimum, mean, median, area, and integrated density at the same time. A similar method can also be performed in ImageJ using the measurement log feature. The measurements were performed in triplicates (n=3) and values were exported to Excel and OriginPro 9.5 to prepare calibration plots.

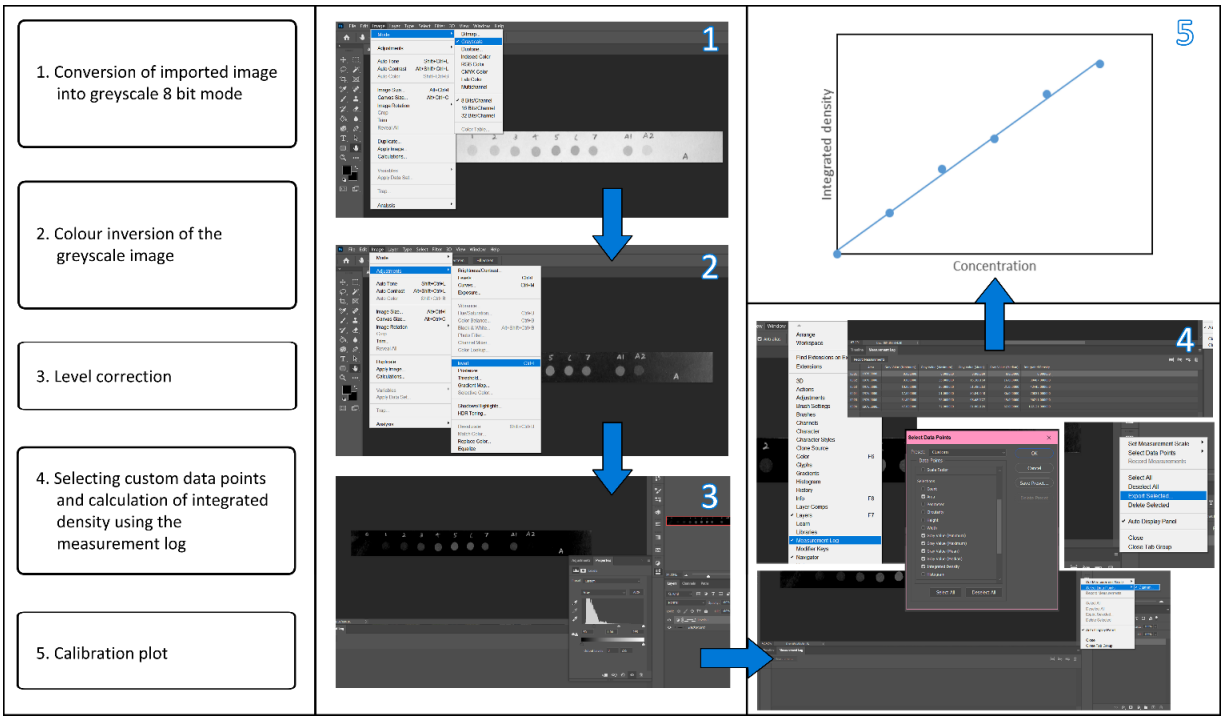

Figure 3: Schematic illustration of quantification of aspirin by the proposed grayscale method in Adobe Photoshop.

2.7. Reference Method

For UV/Visible spectrophotometry, 1000 µg/mL aspirin stock solution was prepared by dissolving 0.1 g of pure aspirin in 5 mL of 1 M NaOH and the solution was hydrolyzed by heating and stirring for 30 minutes. The hydrolyzed solution was transferred to a 100 mL volumetric flask and made up to mark with deionized water. Subsequent standards were prepared in the concentration range of 50-300 µg/mL. The absorbance of violet-colored aspirin solutions was measured at 530 nm and factors like linearity, standard deviation, and percent error were compared with both filter paper spot tests and the 96-microwell plate method.

3. RESULTS AND DISCUSSION

3.1. Preliminary Studies

A chemical reaction involving the formation of an aspirin-iron complex in this method is based on the complexation reaction of Fe(III) to give colored complexes where Fe(III) salts combine with salicylic acid to form a dark violet-colored complex and are used as an identification test for the presence of free salicylic acid present in aspirin. Acetylsalicylic acid hydrolyzes more rapidly in a basic medium than in acidic or aqueous media but optimizing the concentration of sodium hydroxide is important to avoid the formation of $Fe(OH)_3$ precipitates. This was overcome by using an acidified solution of $FeCl_3.6H_2O$ to neutralize excess sodium hydroxide. The absorbance spectra of violet colored aspirin complex showed maximum absorbance at 530 nm as shown in Figure 4, which follows previous studies (17).

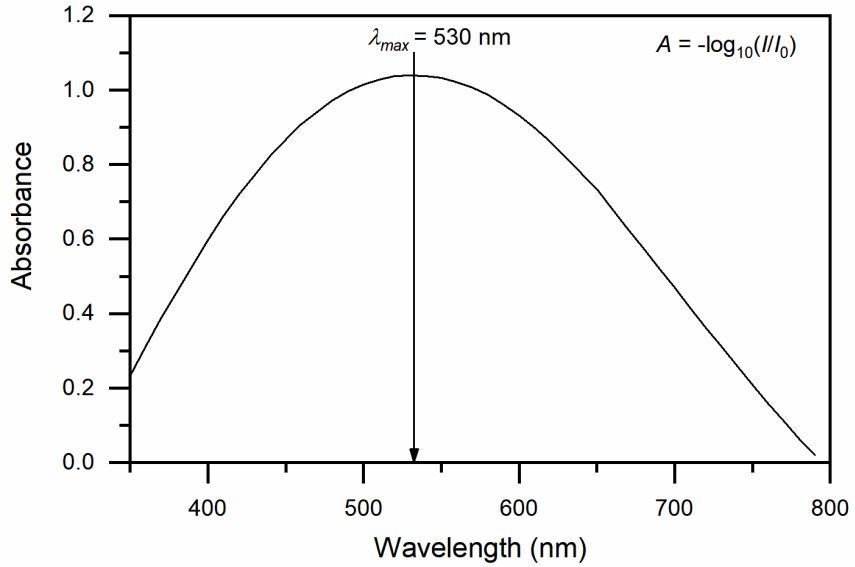

Figure 4: Absorbance spectra of violet colored aspirin-iron complex.

3.2. Effect of Reagent Concentration

The effect of reagent concentration was investigated by keeping the quantity of aspirin the same but varying the concentration of the reagents. Increasing HCl concentration resulted in a decrease in absorbance of the colored complex (Figure 5a) and the physical effect was also evident by observing the discoloration of the aspirin-iron complex. However, varying sodium hydroxide concentration had no considerable effect on absorbance as shown in Figure 5b. An increase in absorbance of the violet-colored complex was observed when changing $FeCl_3.6H_2O$ concentration from 0.1% to 0.5% (Figure 5c). Hence, 0.5% $FeCl_3.6H_2O$ prepared in 0.2 M HCl was selected as the optimum concentration in the present study.

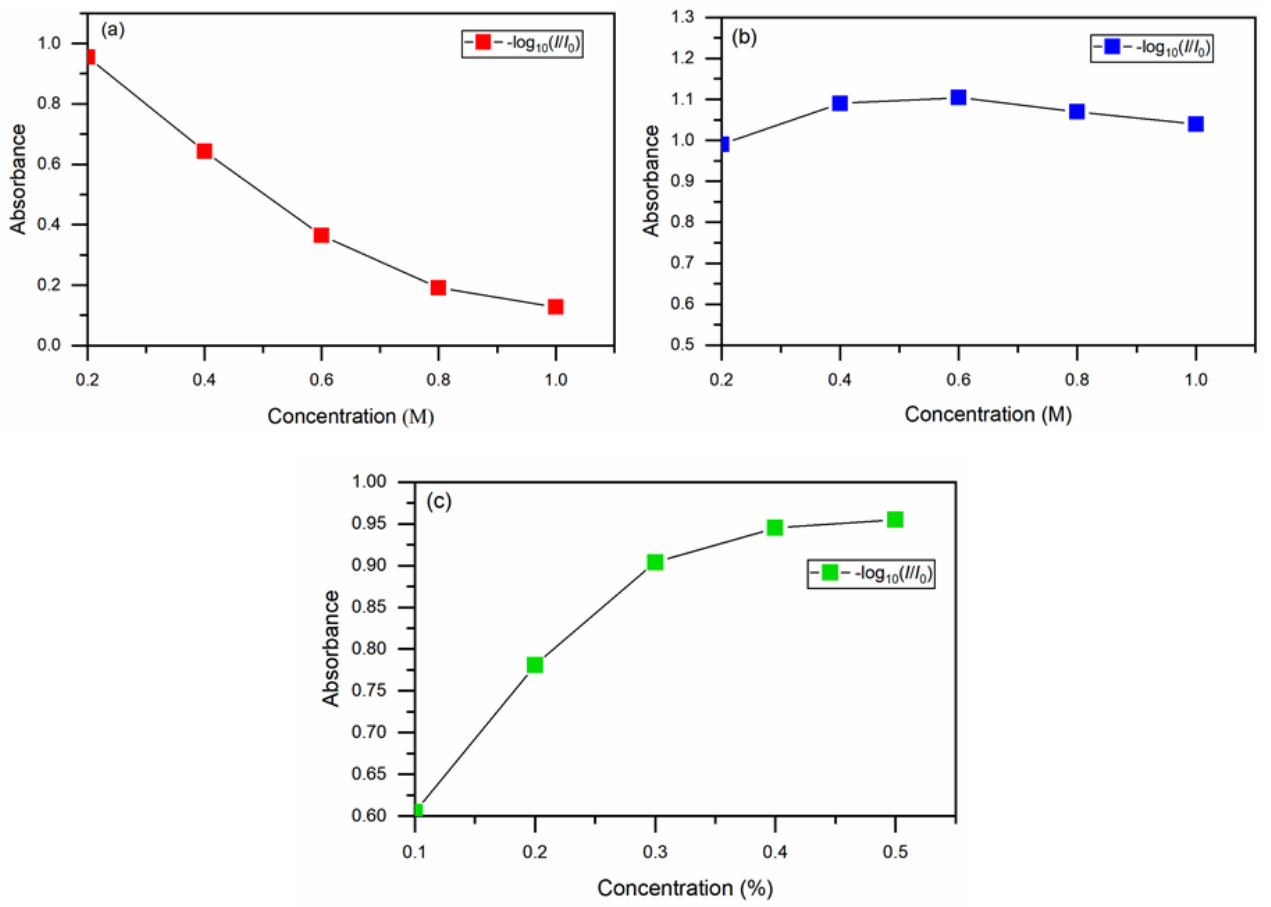

Figure 5: Effect of concentration of (a) HCl, (b) NaOH and (c) $FeCl_3.6H_2O$ on absorbance of colored complex of aspirin.

3.3. Analytical Performance

Quantitative measurements for filter paper aspirin spots were performed in the range of 500-2500 µg/mL. A calibration graph was plotted between the concentration and the integrated density of aspirin spots. To check time variation, aspirin spots were applied on three filter paper strips A, B, and C, and the image was captured after 1 minute, 3 minutes, and 5 minutes respectively and their linearity was compared. Relative standard deviation was found to be less than 2.02% for three sets of spots with R^2 greater than 0.996. Table 2 and Figure 6 compare calibration parameters of images of filter paper aspirin spots that were captured at different time intervals.

Table 2: Effect of time variation on calibration of filter paper aspirin spots.

Calibration parameters	Spots A	Spots B	Spots C	Mean	RSD (%)
Slope	49.305	49.987	55.674	51.655	5.53
Intercept	859.56	2103.0	2130.5	1697.69	34.9
R	0.998	0.999	0.999	0.998	0.05
R^2	0.996	0.998	0.997	0.997	0.08

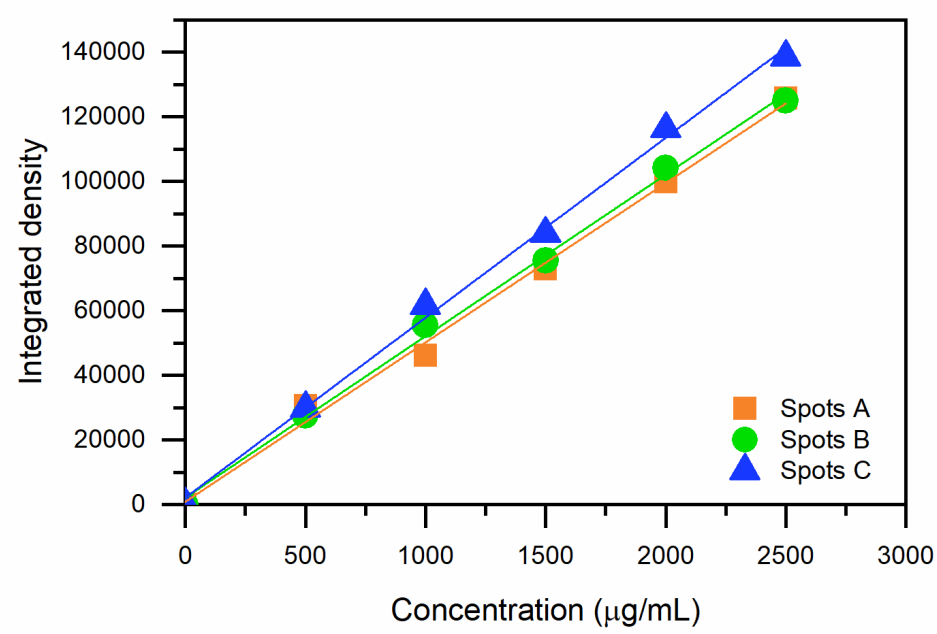

Figure 6: Effect of time variation on filter paper aspirin spots captured by smartphone.

Calibration analysis of 96-microwell plate aspirin also resulted in a good correlation with R^2 of 0.997 and relative standard deviation (RSD, n=3) less than 1.46%. The linear range was found to be the same as that of the reference method (50-300 µg/mL). UV-visible spectrophotometry of aspirin was performed in the range of 50-300 µg/mL and calibration was plotted between concentration versus absorbance of the aspirin solutions. The correlation coefficient (R^2) was found to be 0.996, which was comparable to filter paper spot tests and the 96-microwell plate method. Table 3 and Figure 7 compares the calibration parameters of aspirin filter paper spots tests and 96-microwell plate method with that of the reference. Both filter paper spot tests and the 96-microwell plate method provided precise results using smartphone-camera-based analysis with a correlation coefficient greater than 0.996 and no negative effects of ambient light on the results which were reported by da Silva and Borges (37).

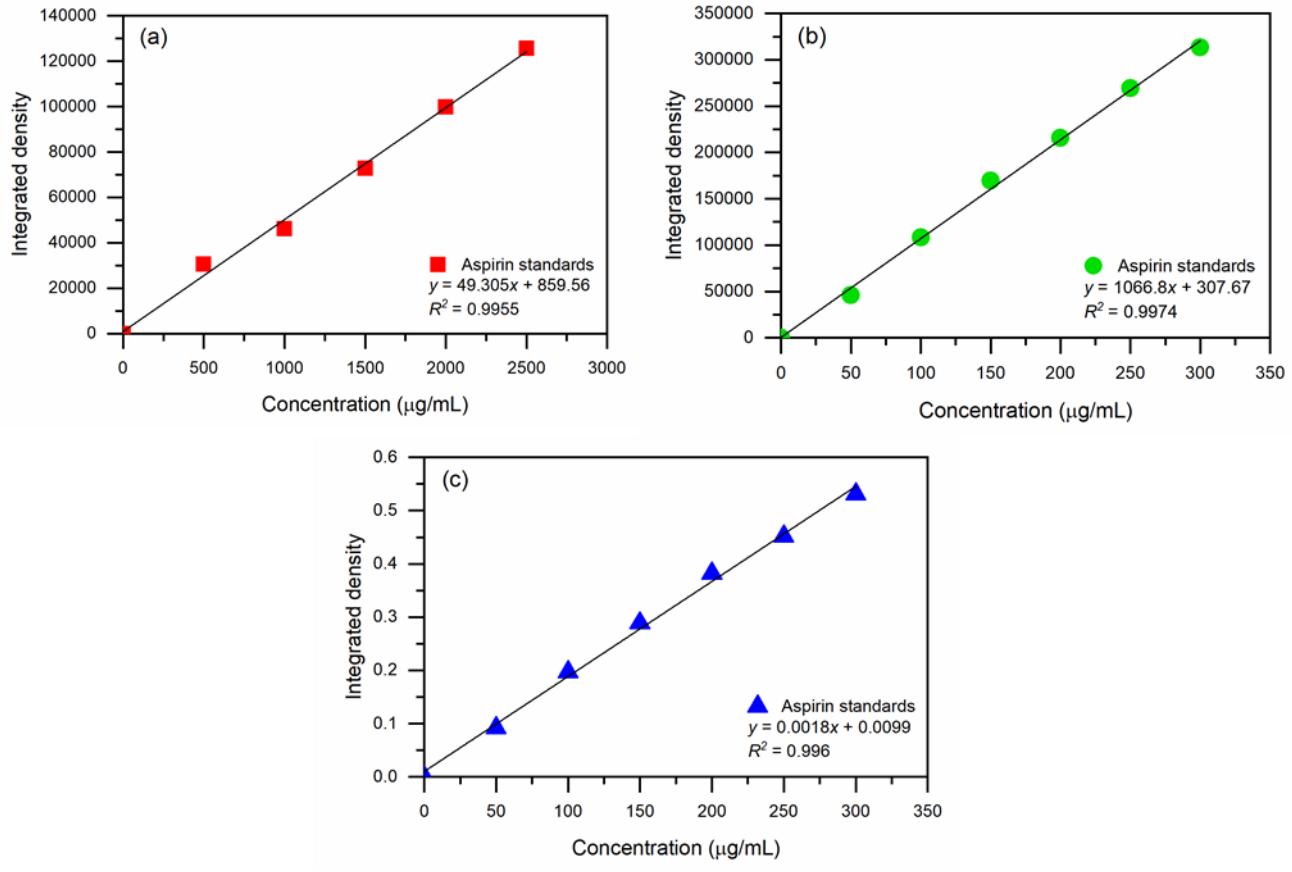

Figure 7: Aspirin calibration by (a) Filter paper spot tests, (b) 96-Microwell plate method and (c) Reference method.

Table 3: Comparison of filter paper spot tests and 96-microwell plate method with the reference method.

Calibration parameters	Filter paper spot tests	96-Microwell plate method	Reference method
Working range (µg/mL)	500-2500	50-300	50-300
Slope	49.305	1066.8	0.0018
Intercept	859.56	307.67	0.0099
R	0.998	0.998	0.998
R^2	0.996	0.997	0.996
Average RSD (%)	0.68	0.61	0.67

3.4. Method Verification

The proposed method was verified by application to aspirin samples in pharmaceutical drugs. Measurements for the mass of aspirin in three samples from different pharmaceutical brands were performed using the proposed grayscale technique for both filter paper and 96-microwell plate method and results were compared with that of spectrophotometry and that of drug label as presented in Table 4. Relative error was found to be less than 3.6% for filter paper spot tests which was better in comparison to the reference method (RE <4.7%). The results also did not differ much from the reference method in the case of 96-microwell plate spot tests (RE <5.2%), which proved that both of the methods could be accurate for smartphone-camera-based analysis by employing the proposed grayscale quantification technique.

Table 4: Determination of aspirin in pharmaceutical formulations.

Sample	Drug label	Reference method		Filter paper spot tests		96-Microwell plate method	
	(mg)	(mg)	RE (%)	(mg)	RE (%)	(mg)	RE (%)
Sample 1	300	298.81	0.4	297.61	0.8	286.02	4.7
Sample 2	300	289.22	3.6	290.65	3.1	284.43	5.2
Sample 3	300	285.77	4.7	289.16	3.6	290.41	3.2

3.5. Comparison of the Grayscale Method with Other Models

The proposed grayscale model was compared with RGB and HSL models using smartphone camera-based analysis for aspirin. RGB model for the analysis of aspirin is based on the absorbance of green color that reflects violet-blue color in the visible spectrum. Therefore, the green component was used for the regression analysis of aspirin spots. It was found that filter paper spot tests posed an advantage over a 96-microwell plate and the linearity was found to be good in the case of all three color models. The correlation coefficient was found to be 0.954 in the case of the RGB model which was better than the reported value (R^2 <0.7) in literature when using smartphones (37). All measurements were performed in Adobe Photoshop using the eyedropper tool. R^2 of 0.958 was observed for the HSL model using the saturation component, while the proposed grayscale model gave the most precise results with a correlation coefficient of 0.996. Figure 8 shows the calibration of aspirin spot tests using RGB, HSL, and grayscale models.

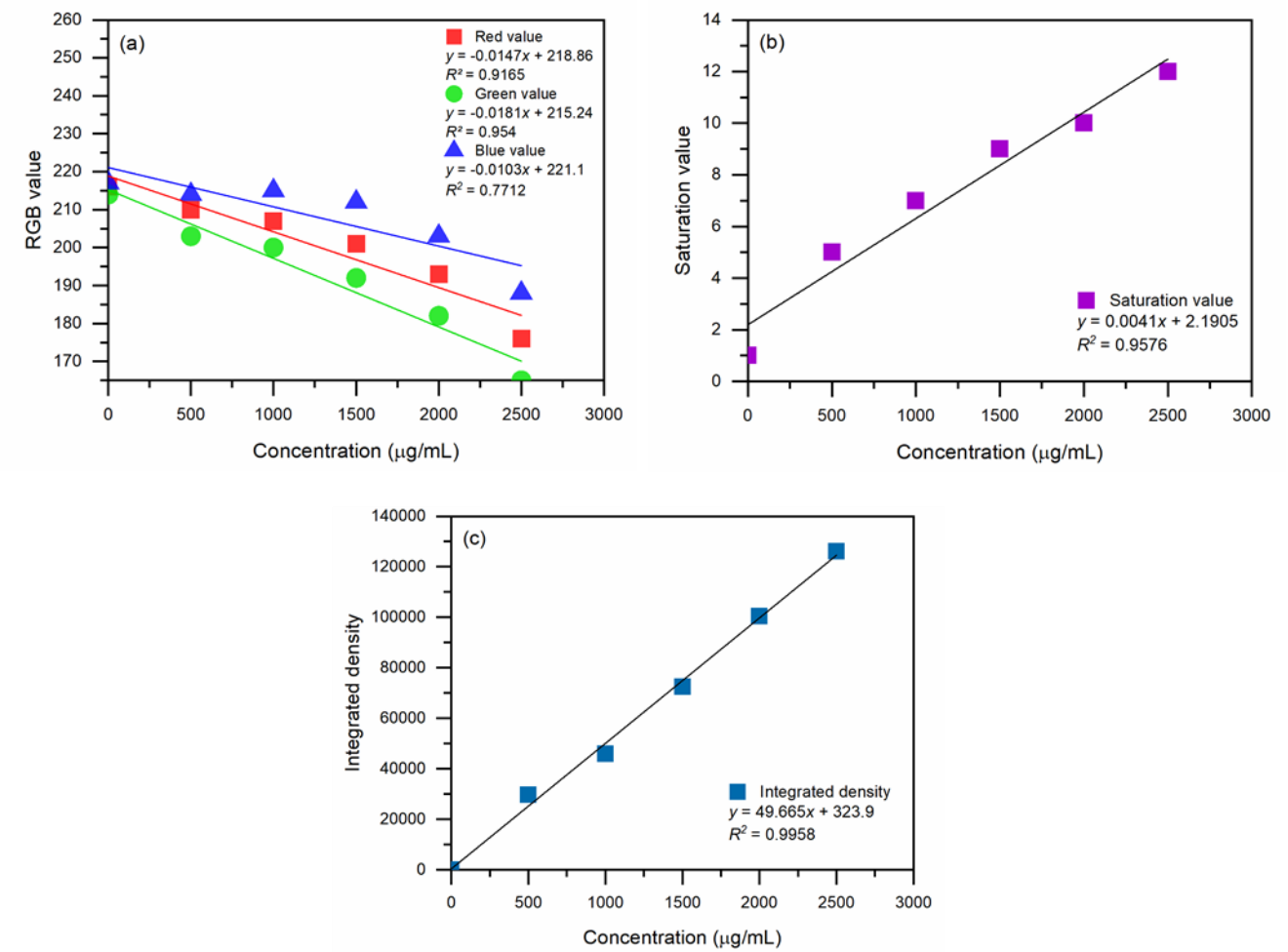

Figure 8: Aspirin calibration by filter paper spot tests using (a) RGB model, (b) HSL model and (c) proposed grayscale model.

The RGB, HSL, and grayscale models were also applied to the 96-microwell plate method using smartphone capture. RGB model gave results similar to that reported in the literature (R^2 =0.792) while better results were observed in the case of the HSL model (R^2 =0.97). The grayscale model gave the best results with R^2 of 0.997. The calibration of the 96-microwell plate method using three color models is shown in Figure 9. Table 5 compares the correlation coefficient values of RGB, HSL, and the proposed grayscale model for both filter paper and 96-microwell plate method.

Table 5: Comparison of RGB, HSL and grayscale models using aspirin spot tests and 96-microwell plate method.

Colour model	Correlation coefficient (R^2)	
	Filter paper spot tests	96-Microwell plate method
RGB	0.954	0.792
HSL	0.958	0.97
Grayscale	0.996	0.997

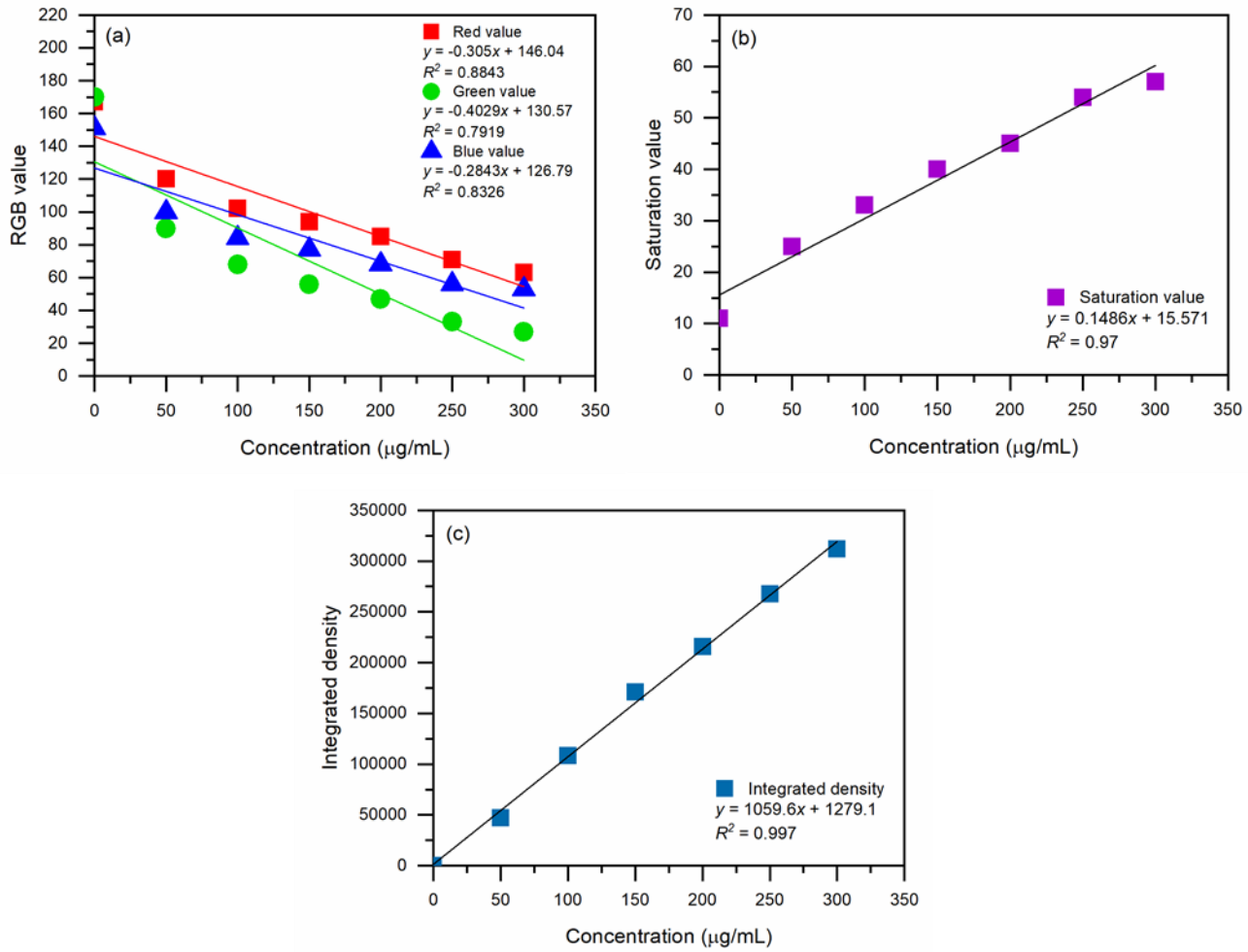

Figure 9: Aspirin calibration by 96-microwell plate method using (a) RGB model, (b) HSL model and (c) proposed grayscale model.

4. CONCLUSION

Smartphone camera-based image scanning densitometry was successfully employed for the quantification of aspirin using filter paper spot tests and the 96-microwell plate method. Both filter paper spot tests and the 96-microwell plate method have their advantages being low cost, simple, portable, and accurate. The present work provided an improved methodology for the analysis of aspirin based on grayscale quantification in Adobe Photoshop. The precision of the proposed technique was determined by comparison of spot tests and 96-microwell plate method with UV-visible spectrophotometry, resulting in a correlation coefficient (R^2) greater than 0.996. Three commercial samples of aspirin were assayed and compared with that of the drug label with relative errors lower than 5.2% for the 96-microwell plate method and less than 3.6% for a spot test method. Regression analysis of the proposed grayscale method was also compared with that of RGB and HSL models used in the literature. The grayscale method accounted for better results for both filter paper aspirin spot tests and the 96-microwell plate method and can be recommended for future research in pharmaceutical, medical, and other analytical fields.

5. CONFLICT OF INTEREST

The authors declare that there are no conflicts of interest to disclose.

6. ACKNOWLEDGMENTS

This work was not acknowledged by any source.

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