

Analytical Letters

ISSN: 0003-2719 (Print) 1532-236X (Online) Journal homepage: www.tandfonline.com/journals/lanl20

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To cite this article: Tülay Borahan Kustanto, Buse Tuğba Zaman, Ümmügülsüm Polat Korkunç, Mehmet Selim Gel & Sezgin Bakırdere (11 Jun 2025): Simple Determination of Cystine in Artificial Urine and Drug Matrices Using Digital Image Colorimetry, Analytical Letters, DOI: 10.1080/00032719.2025.2517118

To link to this article: https://doi.org/10.1080/00032719.2025.2517118

Published online: 11 Jun 2025.

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ANALYTICAL LETTERS
https://doi.org/10.1080/00032719.2025.2517118

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Simple Determination of Cystine in Artificial Urine and Drug Matrices Using Digital Image Colorimetry

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ABSTRACT

Smartphone-based digital image colorimetry was developed for the determination of cystine in pharmaceutical formulations and artificial urine samples. A lab-made colorimetric box was designed for capturing the digital images of sample solutions using a smartphone. The developed method employs a simple colorimetric method called nitroprusside test which specifically targets free -SH groups. Cystine was reduced to cysteine before the colorimetric test was applied. The color intensity is inversely related to the concentration of the analyte. The best linearity was obtained with the G color channel. Digital image processing was performed using the RGB Color Detector mobile application to obtain analytical data. The developed method exhibited good linearity in the range of 29.7–00.3 mg/kg. The limits of detection and quantification were 9.7 mg/kg and 32.3 mg/kg, respectively. The percent recoveries were 104.2 ± 7.8% and 108.7 ± 4.6%, which proved the accuracy and applicability of the developed method.

ARTICLE HISTORY
Received 1 April 2025
Accepted 4 June 2025

KEYWORDS
Cystine; determination; digital image colorimetry; smartphone; spectrophotometry; urine

Introduction

Amino acids are known to be the main constituents of metabolism. They are the primary components of proteins and are also precursors to a number of hormones and neurotransmitters. They regulate gene expression, cellular metabolism, and signaling (Ferré et al. 2019; Joyce et al. 2016). Therefore, an optimum concentration of amino acids in the body is crucial for maintaining normal homeostasis (Joyce et al. 2016). A deficiency in enzymes linked with a metabolic pathway of amino acids leads to an abnormal accumulation of amino acids and their precursors in the body. In consequence, amino acid levels increase in urine and blood (Namera et al. 2002). Minor alterations in amino acid levels have been linked to metabolic dysregulations, and some fluctuations have been suggested as possible biomarkers for diseases (Ferré et al. 2019) such as diabetes (Bi and Henry 2017), cancer (Gao et al. 2016), inborn errors of metabolism (Waterval et al. 2009) and cystinuria (Öztürk Er, Özbek, and Bakırdere 2021).

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Cystine is an amino acid dimer of two cysteine molecules that are linked together by a disulfide bond (Robert et al. 2014). Cystine plays an important role in various essential physiological processes, such as detoxification, metabolism, and protein synthesis (Wear and Keevil 2005). It is a crucial constituent of hair and is thought to stimulate hair growth and inhibit hair loss (Hengl et al. 2018). Pharmaceutical tablets containing cystine are prescribed in China for the treatment of hair loss after illness and postpartum, as well as an additional therapy for chronic hepatitis (Kuo et al. 2023). Cystine is known to be excreted *via* the kidneys (Wear and Keevil 2005). Elevated concentrations of cystine in urine can be an indicator of cystinuria which is a rare hereditary disorder. Cystine stones are produced when the patients with cystinuria have excessive cystine excretion from the kidneys into urine (Kuo et al. 2023). Thus, it is important to develop analytical techniques to determine cystine qualitatively and quantitatively in urine samples and pharmaceutical formulations.

Several analytical techniques have been reported for the determination of cystine in various samples such as high-performance liquid chromatography (Varzaru et al. 2013; Zhang et al. 2014), liquid chromatography-tandem mass spectrometry (Dogan et al. 2018; Seiwert and Karst 2007), spectrophotometry (Garcia Alvarez-Coque et al. 1988) and capillary electrophoresis (Ivanov et al. 2024). Colorimetric methods such as the Millon, Xanthoproteic, Hopkins-Cole, Sakaguchi, and Nitroprusside tests are used for the qualitative determination of amino acids (Asthana et al. 2019; Mim, Tabassum, and Hashem 2018; Brady et al. 2017). The nitroprusside test is a colorimetric technique that is widely used as a qualitative test to identify the presence of cysteine in various samples. A chromogenic agent, namely sodium nitroprusside reagent, and ammonia solution is used in the experimental procedure (Brady et al. 2017; Subrotoet al. 2020 –). The free -SH group, in cysteine is known to react with nitroprusside to form a red-colored compound (Subroto et al. 2020).

In today's modern society, on-site analysis is becoming increasingly significant (Fan et al. 2021). In parallel with the recent advancement of imaging, digital image-based colorimetry has emerged as an alternative for fast and simple determinations on-site in the field of analytical chemistry (Firdaus et al. 2014). It has been used widely for the qualitative and quantitative determinations of inorganic and organic analytes in a wide range of sample types (Abdullahi et al. 2023; Ismail et al. 2023; Sáez-Hernández et al. 2022; Choodum, Sriprom, and Wongniramaikul 2019). Digital image-based colorimetry is a colorimetric analysis based on the digitization of images obtained from various image capture devices, such as mobile phones (Firdaus et al. 2022), digital cameras (Lapresta-Fernández and Capitán-Vallvey 2011), webcams (Thajee et al. 2018), and hand scanners (Fan et al. 2021; Kompany-Zareh and Mirzaei 2004). Smartphones are mostly preferred in digital image-based colorimetry compared to webcams, scanners and digital cameras due to their portability, the prevalence of mobile applications, rapid increment in usage, and remarkable advancements of camera functions (Masawat, Harfield, and Namwong 2015; Anconi, Brito, and Nunes 2022).

In the literature, colorimetric methods have been reported for the determination of amino acids and cystine in biological and pharmaceutical samples using unmodified gold nanoparticles (Lu et al. 2014; Rastegarzadeh and Hashemi 2015), silver nanoparticles (Khatun et al. 2024) and spectrophotometry (Aly 2008). These techniques require

a synthesis of the nanoparticles and specialized instrumentation for colorimetric detection. On the other hand, smartphone-based digital image colorimetry has several advantages such as ease of handling, reduced cost, field portability, fast detection, development of miniaturized and automated systems, visual qualitative feedback and proposing specific procedures for on-site analysis (Porto et al. 2019; Wongniramaikul, Limsakul, and Choodum 2018). This method is an effective alternative to conventional techniques in laboratories with limited equipment and resources (Chan, Tan, and Wu 2017).

In digital image-based colorimetry, the first step is to capture the digital images using an image-capturing device. The second is to process the digital images into color spaces using specific image processing software to obtain analytical data (Fan et al. 2021; Zhao et al. 2023). Several color systems have been suggested to create a three-dimensional coordinate space that represents each color with a single point to enhance a standardized specification. CMYK (Cyan, Magenta, Yellow and black), L*a*b*, RGB (Red, Green, and Blue), XYZ, HSV, and Grey models are among the most often used color systems (Jain et al. 2022). The RGB color space is widely acknowledged as the most broad and accepted color space system due to its advantages such as the easy measurement of individual data in a user-friendly way and a useful representation of images (43). Image processing software can break down any color into its three primary colors ranging from 0 to 255 to represent brightness. The RGB color channels are directly related to color brightness (Lin et al. 2018). The color intensities obtained from the RGB data are correlated with the concentration of the analytes and used as the analytical data to obtain calibration graphs (Wongniramaikul, Limsakul, and Choodum 2018).

In this study, a smartphone-based digital image-based colorimetric method was developed for the rapid quantification of cystine in pharmaceutical and artificial urine samples. The proposed method is simple, fast and no professional personnel or expensive instruments are required. The developed method was applied to pharmaceutical tablets and artificial urine samples to evaluate the method's applicability and accuracy. Acetylcysteine and cysteine determination in pharmaceutical formulations using a smartphone-based digital image colorimetric method has been reported in the literature by our research group (Şahin et al. 2024). This current study focuses on the determination of cystine after reduction reaction using the nitroprusside test for thiol detection, whereas our previous reported study is for the direct determination of free thiol-containing compounds (acetylcysteine and cysteine).

Experimental

Apparatus and instruments

The digital images were captured by a Huawei smartphone (ANE-LX1, Android 9.0) which was equipped with a dual camera (16-megapixel, f/2.2 lens aperture, 2-megapixel sensor for depth) and 5.84-inches touchscreen display with a resolution of 1080 × 2280 pixels. RGB Color Detector smartphone application was used for data processing. A portable rectangular colorimetric box (25.0 × 20.0 cm) was designed in the laboratory from Styrofoam with a white inner surface. The exterior part of the colorimetric box was tapped off with black tunnel tape to isolate any surrounding light and create a

completely dark environment. A small hole was drilled in the front side of the box where the smartphone was positioned to capture the digital images of the solutions that were placed in a 1.0 cm plastic cell. The distance from the smartphone to the sample/standard solutions was kept constant to ensure image reproducibility. The inner bottom part of the colorimetric box was covered with white paper and the plastic cell was positioned on the white paper sheet. A battery-powered 1.5 m strip of white LED lamp (12 volts) was attached to the top surface of the box as the light source. In order to obtain a uniform distribution of light, two white papers were placed over the LED lamp.

A Mettler Toledo pH/ORP meter was used for pH measurements. An ultrasonic cleaner (Runyes Ultrasonic Cleaner CLEAN-01) was used for the ultrasonication process. An electronic balance (Shimadzu) was used for all weighing procedures.

Reagents and chemicals

Analytical grade reagents were used to prepare the solutions. Deionized water obtained from an ELGA PureLab Flex 3 deionized water system was used to prepare the aqueous solutions. The cystine standard used in this study was obtained from Sigma-Aldrich. The stock cystine solution was prepared in 100 mM hydrochloric acid. Calibration and working standard solutions were prepared in 25 mM hydrochloric acid by diluting with deionized water from the stock standard solution. Sodium borohydride ($NaBH_4$, Carlo Erba) was prepared daily before use in deionized water. Sodium nitroprusside dihydrate obtained from Sigma-Aldrich was prepared in deionized water. Glacial acetic acid, hydrochloric acid (37%), ammonia solution (25%), sodium chloride, potassium chloride, sodium dihydrogen phosphate dihydrate and sodium hydroxide were obtained from Merck. Urea and creatinine were purchased from Sigma-Aldrich.

Preparation of the artificial urine and pharmaceutical formulations

The recovery experiments were carried out on artificial urine samples. The study reported by Reetu et al. Reetu, Kar, and Panda (2015) was slightly modified to prepare the artificial urine samples. 6.0 g urea was dissolved in 250 mL of deionized water. Then, 2.5 g of sodium chloride, 1.5 g of potassium chloride and 1.6 g of sodium dihydrogen phosphate dihydrate were added and mixed until a clear solution was obtained. The pH of the resulting solution was fixed to 6.55 using 1.0 M sodium hydroxide solution. Finally, 250 mL of this solution was mixed with 0.50 g creatinine.

A commercially available tablet containing cystine as one of the active pharmaceutical ingredients was used for the real sample application. Five tablets were weighed, and their average weight was measured. Afterward, they were ground into a fine powder using a mortar. The stock sample solution was prepared in 0.10 M hydrochloric acid and further diluted with deionized water.

Nitroprusside test by smartphone-based digital-image colorimetric method

The nitroprusside test used in this study is to identify cysteine. In order to apply this method to samples containing cystine, a reduction experiment was conducted to reduce

cystine into cysteine. $NaBH_4$ was used as the reducing agent for the disulfide bond of cystine. The reduction experiment reported in the literature (Takahashi and Ooi 1976) was modified and optimized. The experiment was performed as follows. 2.0 mL of cystine standard was mixed with 1.0 mL of 5.0% $NaBH_4$ and kept in the ultrasonic bath for 90 min at 37 °C. The excess $NaBH_4$ was decomposed by adding 0.10 mL of glacial acetic acid at 38 °C and sonicated for 45 min. Next, the nitroprusside test was applied. The nitroprusside test proposed in the literature (Subroto et al. 2020) was modified in this study. 0.50 mL of ammonia solution (25%) was added to 2.0 mL of reduced standard/sample solution. For the final step, the colorimetric reagent (0.25 mL of 4.80% sodium nitroprusside dihydrate solution) was added to the resulting solution. The colorimetric measurement was conducted immediately. The color channel was selected by constructing calibration graphs to determine which recorded the best linearity. The G channel recorded the best linearity.

Results and discussion

Optimization studies were carried out for each parameter of the nitroprusside test and the reduction reaction to maximize the reaction efficiencies and lower the detection limit. For this context, nitroprusside and reduction reaction parameters including the concentration and volume of sodium nitroprusside dihydrate, volume of ammonia solution, concentration and volume of sodium borohydride solution, concentration of sodium hydroxide, ultrasonication periods, and volume of acetic acid were optimized. These reaction conditions were optimized by changing one parameter at a time while keeping others constant. In the optimization studies, the difference observed in color intensities between blank and reaction solutions was considered: the parameter with the maximum difference was chosen as the optimum value.

Reduction reaction optimization

The reduction of cystine to cysteine before the nitroprusside test is mandatory since the nitroprusside test is for cysteine. The reductive cleavage of the cystine disulfide bond was carried out by reaction with sodium borohydride. In the reduction reaction, disulfides are converted to their thiol derivatives with a free thiol group (Kuśmierek and Bald 2008).

The first optimization study for the reduction reaction was the volume of sodium borohydride. The effect of sodium borohydride volume was evaluated in the range of 0.50–1.0 mL and compared with the results where no sodium borohydride solution was added. The color did not change to red without the addition of sodium borohydride. As can be seen in Figure 1, the intensity difference between the blank increased with the sodium borohydride volume. Therefore, 1.0 mL of 5.0% sodium borohydride was selected as the optimum condition throughout the analyses.

For the next optimization step, the concentration of sodium borohydride on the reaction efficiency was investigated. For this purpose, sodium borohydride solutions were prepared in 2.0% sodium hydroxide at the concentrations of 2.0%, 5.0%, 7.5% and 10% (w/w) and used in the reduction reaction. The results increased after 2.0% and no

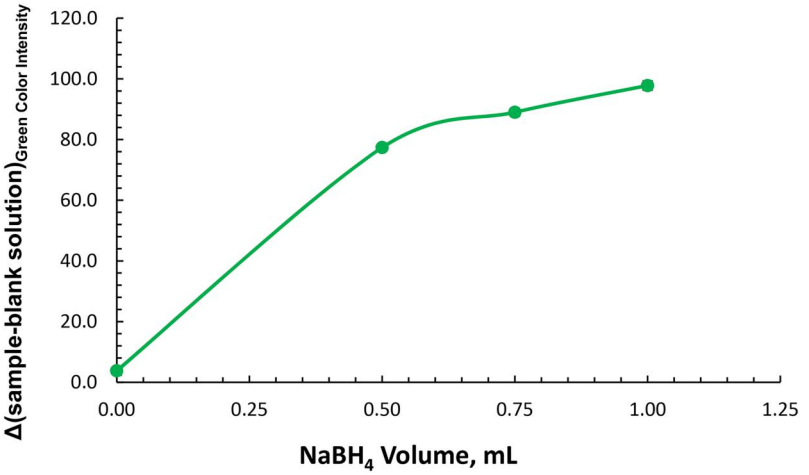


Figure 1. The effect of sodium borohydride volume. (2.0 mL of 50 mg/L cystine standard, 5.0% sodium borohydride in 2.0% sodium hydroxide, 60 min ultrasonication at 37 °C, 200 µL of acetic acid, 30 min ultrasonication at 38 °C, 2.0 mL of reduced cystine standard solution, 1.0 mL of ammonium hydroxide solution and 0.50 mL of 4.80% sodium nitroprusside solution).

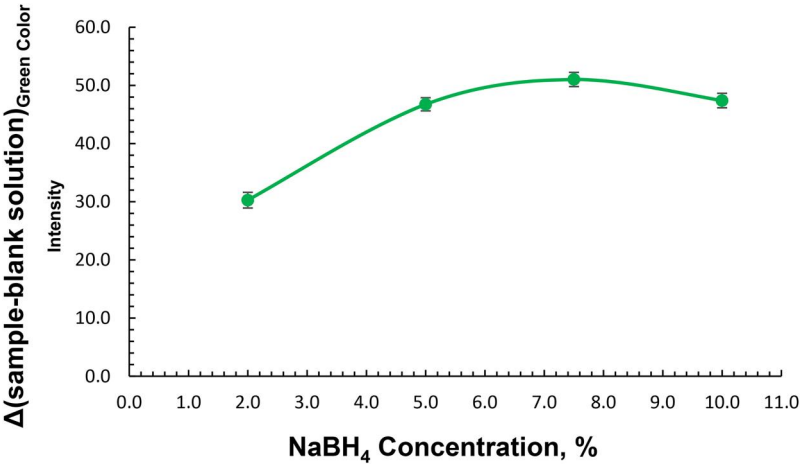


Figure 2. The effect of sodium borohydride concentration. (2.0 mL of 35 mg/L cystine standard, 1.0 mL of sodium borohydride in 2.0% sodium hydroxide, 60 min ultrasonication at 37 °C, 200 µL of acetic acid, 30 min ultrasonication at 38 °C, 2.0 mL of reduced cystine standard, 1.0 mL of ammonium hydroxide solution, and 0.50 mL of 4.80% sodium nitroprusside solution).

significant change was observed after 5.0% (Figure 2). Thus, 1.0 mL of 5.0% sodium borohydride was chosen as the optimum to decrease the chemical consumption.

$NaBH_4$ is usually prepared in sodium hydroxide to increase its stability (Bodur et al. 2023). The optimization of sodium hydroxide concentration in which the sodium borohydride solution is prepared was also performed. In this context, 5.0% sodium borohydride was prepared in 0.10, 0.20, 0.50, 1.0 and 2.0 M sodium hydroxide and used in the reduction reaction. Freshly prepared 5.0% sodium borohydride solution was also prepared and compared with the results. According to the results, no color change was observed when 1.0 and 2.0 M of sodium hydroxide were used. As can be seen in

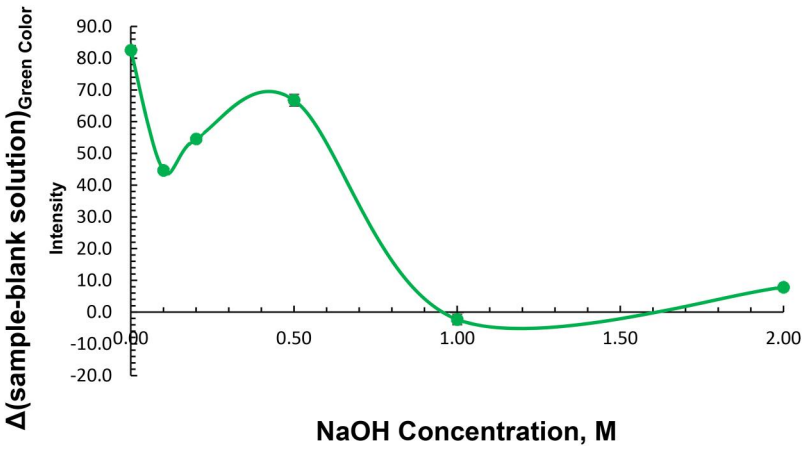


Figure 3. The effect of sodium hydroxide concentration. (2.0 mL of 50 mg/L cystine standard, 1.0 mL of 5.0% sodium borohydride, 90 min ultrasonication at 37 °C, 100 µL of acetic acid, 45 min ultrasonication at 38 °C, 2.0 mL of reduced cystine standard, 0.50 mL of ammonium hydroxide solution, and 0.25 mL of 4.80% sodium nitroprusside solution).

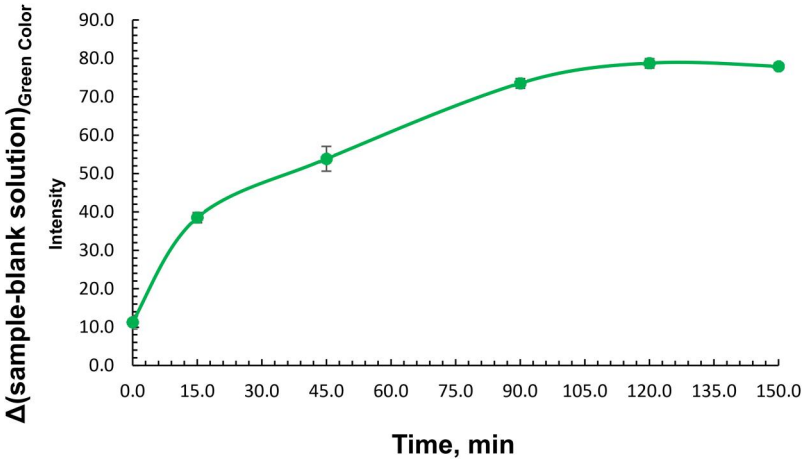


Figure 4. The effect of ultrasonication period after sodium borohydride addition. (2.0 mL of 50 mg/L cystine standard, 1.0 mL of 5.0% sodium borohydride in 2.0% sodium hydroxide, 150 µL of acetic acid, 30 min ultrasonication at 38 °C, 2.0 mL of reduced cystine standard, 1.0 mL of ammonium hydroxide solution and 0.50 mL of 4.80% sodium nitroprusside solution).

Figure 3, negative intensity values were observed at 1.0 M NaOH and a drastic decrease occurred at 2.0 M NaOH. Freshly prepared 5.0% sodium borohydride solution had the highest intensity difference between the blank solution. Thus, fresh sodium borohydride solution was used in the following experiments.

After the sodium borohydride was added to the standard solutions, an agitation procedure with an ultrasonic bath was applied to investigate its effect on the reduction efficiency. The impact of the ultrasonication period was studied between 0.0–150 min at 37 °C. The results increased up to 120 min and reached a plateau. The results (Figure 4)

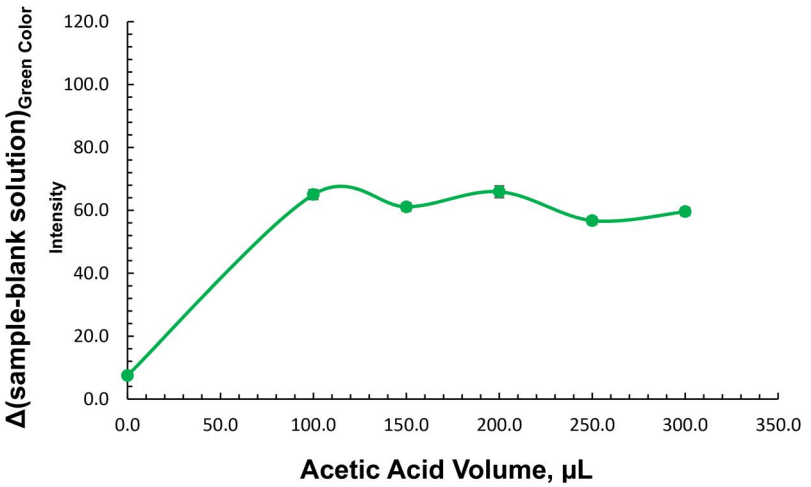


Figure 5. The effect of acetic acid volume. (2.0 mL of 50 mg/L cystine standard, 1.0 mL of 5.0% sodium borohydride in 2.0% sodium hydroxide, 90 min ultrasonication at 37 °C, 30 min ultrasonication at 38 °C, 2.0 mL of reduced cystine standard, 1.0 mL of ammonium hydroxide solution and 0.50 mL of 4.80% sodium nitroprusside solution).

between 90 min and 120 min were not significantly different; therefore, 90 min was selected as the optimum to decrease the ultrasonication period.

Following the reduction reaction, excess sodium borohydride was decomposed by the addition of acetic acid. The volume of acetic acid was optimized to study its effect on the reaction. The optimum volume of acetic acid was tested by adding glacial acetic acid in 0.0, 100, 150, 200, 250, and 300 µL volumes to the reduced standard solutions. The obtained results (Figure 5) revealed that 100 µL of acetic acid was enough to decompose the excess sodium borohydride. Furthermore, a color change was not recorded for the solutions without acetic acid addition. For this reason, for subsequent analyses, 100 µL of glacial acetic acid was used.

Ultrasonic agitation was applied after the acetic acid addition to facilitate the decomposition. The period of ultrasonication was tested between 0.0–60 min at 38 °C. According to the obtained results, the ultrasonication period was selected to be 45 min, where the maximum intensity difference was obtained with the blank solution (Figure 6).

Nitroprusside test optimization

The optimum parameters of the nitroprusside reaction in this study were taken from our previous study (Şahin et al. 2024). The reaction time was previously optimized in which sample solutions were measured after waiting periods of 0, 30, 60, and 120 s following nitroprusside addition. The best results were obtained when image acquisition was done immediately after the reaction completion. The volumes of ammonium hydroxide and sodium nitroprusside were optimized again in this study. In the sodium nitroprusside volume optimization, volumes of 4.80% sodium nitroprusside solution were tested between 0.25–1.0 mL. According to the results (Figure 7), the highest

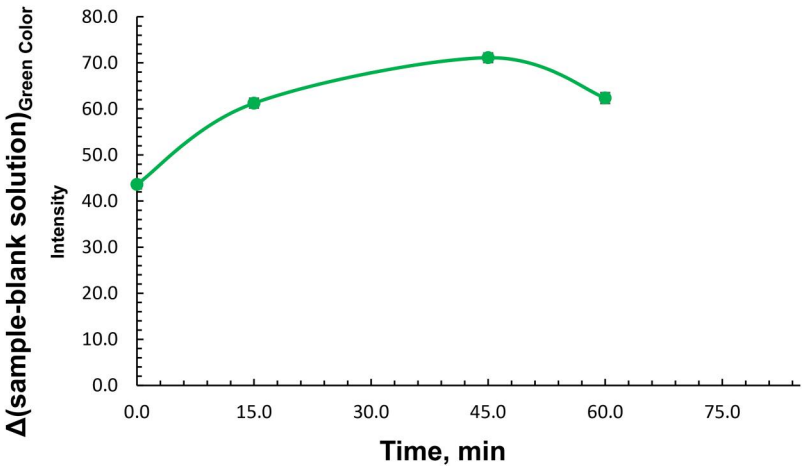


Figure 6. The effect of ultrasonication period after acetic acid addition. (2.0 mL of 50 mg/L cystine standard, 1.0 mL of 5.0% sodium borohydride in 2.0% sodium hydroxide, 90 min ultrasonication at 37 °C, 100 µL of acetic acid, 2.0 mL of reduced cystine standard, 0.50 mL of ammonium hydroxide solution and 0.50 mL of 4.80% sodium nitroprusside solution).

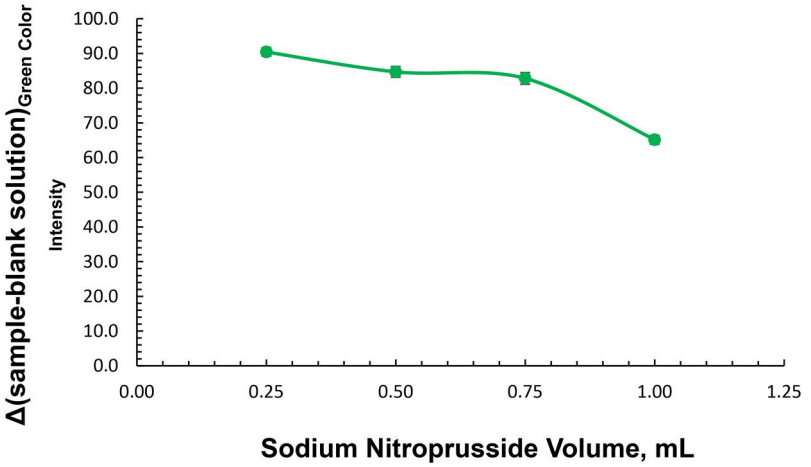


Figure 7. The effect of sodium nitroprusside volume. (2.0 mL of 50 mg/L cystine standard, 1.0 mL of 5.0% sodium borohydride in 2.0% sodium hydroxide, 90 min ultrasonication at 37 °C, 100 µL of acetic acid, 45 min ultrasonication at 38 °C, 2.0 mL of reduced cystine standard, 0.50 mL of ammonium hydroxide solution and 4.80% sodium nitroprusside solution).

difference between the blank was observed with 0.25 mL of sodium nitroprusside solution (4.80%); therefore, it was selected to be optimum.

Afterward, the effect of 25% ammonium hydroxide volume was studied over the range of 0.50-2.0 mL. The results (Figure 8) were not significantly different from each other; therefore, 0.50 mL of 25% ammonium hydroxide solution was selected as the optimum to reduce the solvent consumption. Additionally, the nitroprusside reaction was also conducted without adding ammonium hydroxide to investigate its effect on the reaction. No color change was observed without the addition of ammonium hydroxide,

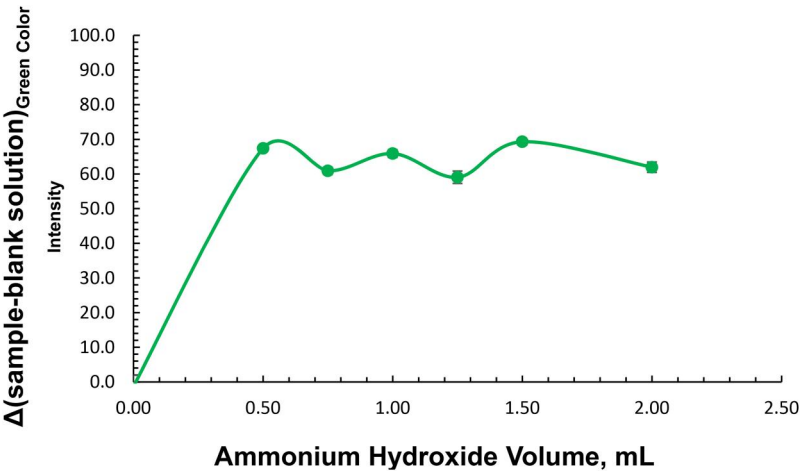


Figure 8. The effect of ammonium hydroxide volume. (2.0 mL of 50 mg/L cystine standard, 1.0 mL of 5.0% sodium borohydride in 2.0% sodium hydroxide, 90 min ultrasonication at 37 °C, 100 µL of acetic acid, 30 min ultrasonication at 38 °C, 2.0 mL of reduced cystine standard, and 0.50 mL of 4.80% sodium nitroprusside solution).

Table 1. Optimum conditions of the developed smartphone-based digital-image colorimetry method.

Parameter	Optimization range	Optimum parameters
Sodium borohydride concentration/volume	2.0–10%, 0.0–1.0 mL	5.0% w/w, 1.0 mL
Ultrasonication period	0.0–150 min	90 min
Glacial acetic acid volume	0.0–300 µL	100 µL
Ultrasonication period	0.0–60 min	45 min
Sodium nitroprusside concentration/volume	0.25–1.0 mL	4.80% w/w, 0.25 mL
Ammonia solution (25%) volume	0.50–2.0 mL	0.50 mL

highlighting its significance in the reaction. Table 1 is the summary of the optimal conditions.

Analytical figures of merit of smartphone-based digital-image colorimetry

The analytical performance of the smartphone-based digital image colorimetry was evaluated in terms of linearity, precision, coefficient of determination (R^2), accuracy, limit of detection (LOD) and limit of quantification (LOQ). Under the optimum conditions, the calibration plot of cystine was constructed using the G color channel from the reduced cystine standard solutions. The intensities were obtained from the four areas of each digital image. Average green color intensities were used for the calibration graphs. The data recorded were converted to log base 10. The G color channel was inversely related to the concentration of the analyte. The calibration graph was linear in the range of 29.7-200.3 mg/kg with a R^2 of 0.9918. The detection limit was calculated based on 3SD/m and limit of quantification based on 10SD/m, where, SD is the standard deviation of the lowest concentration of the calibration plot and m is the slope of the linear calibration equation. The equation of the

Table 2. Results of the real sample and recovery.

Sample	Labeled amount/Spiked concentration	Calculated amount/ %Recovery
Tablet	500 mg	471.2 ± 1.2 mg
Artificial Urine	50.8 mg/kg	104.2 ± 7.8%
	77.9 mg/kg	108.7 ± 4.6%

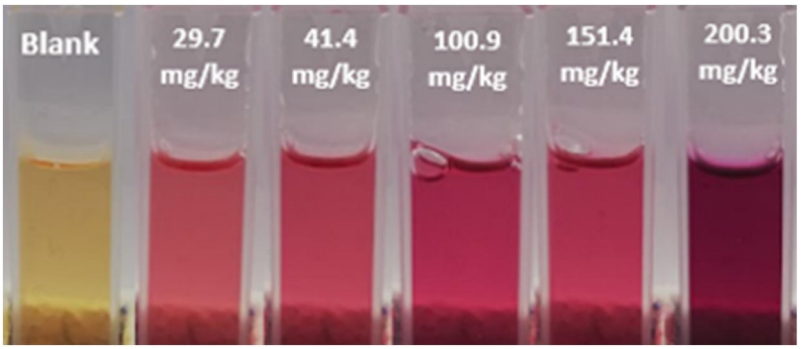


Figure 9. Photographs of the cystine standards and blanks.

calibration plot is $y = -0.0035x + 1.9367$. The LOD and LOQ were 9.7 and 32.3 mg/kg, respectively. Precision, expressed in terms of relative standard deviation (RSD) is 0.6%.

Real sample analysis

The developed colorimetric method was also applied to real samples to evaluate its accuracy. In this context, cystine was determined quantitatively in a tablet pharmaceutical formulation to determine whether the amount of cystine in the tablets matched the label claim. According to the label claim, one tablet contains 500 mg of cystine. The samples were prepared as described above. Under the optimum conditions, the sample solutions were analyzed and their cystine concentration was calculated *via* the external calibration technique. The average cystine amount per tablet was 471.2 ± 1.2 mg (Table 2). The result obtained was significantly close to the label claim, demonstrating the method's accuracy.

Recovery studies were also performed in spiked artificial urine samples to verify the accuracy and applicability to real samples. Artificial urine samples were spiked at seven concentration levels and analyzed under the optimal conditions with the standard solutions. The recoveries calculated using the external calibration method were not close to 100%, which indicated that the accuracy of the results was affected by the matrix.

Hence, matrix matching calibration was utilized in order to improve the accuracy. A matrix-matched calibration plot was constructed using the G color channel intensities of spiked artificial urine samples. The recovery results calculated for the two concentrations were 104.2 ± 7.8% and 108.7 ± 4.6% (Table 2). Hence, the matrix matching calibration strategy improved the accuracy. The recovery studies verified the accuracy and applicability of the developed method to the urine samples for the quantification of cystine. Figure 9 shows the photographs of the cystine standards and blank utilized for the calibration plot.

Conclusion

A smartphone-based digital-image colorimetric method is reported for cystine determination in pharmaceutical formulations and artificial urine samples. A lab-made colorimetric box was prepared and combined with a smartphone for the digital image acquisition of the samples. The developed method exhibits advantages such as portability, ease of operation, environmental friendliness and affordability compared to the literature for the determination of cystine. There is no need for skilled and experienced operators to perform this method. These advantages make this method highly useful in low-income laboratories where expensive instruments are limited. The real sample application and recovery studies proved the method's accuracy and applicability to the real samples.

Authors' contributions

Tülay Borahan Kustanto: Data curation, Formal analysis, Investigation, Methodology, Validation, Visualization, Writing – original draft. Buse Tuğba Zaman: Formal analysis, Methodology, Validation, Writing – original draft. Ümmügülsüm Polat Korkunç: Formal analysis, Methodology, Validation, Writing – orginal draft. Mehmet Selim Gel: Formal analysis, Methodology, Writing – orginal draft. Sezgin Bakırdere: Conceptualization, Data curation, Investigation, Methodology, Supervision, Validation, Writing– review & editing.

Disclosure statement

No potential conflict of interest was reported by the author(s).

Data availability statement

Data will be made available upon reasonable request from corresponding authors.
The authors declare that they have no conflicts of interest.

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