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Cover Page Footnote

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Microfluidic Paper-Based Analytical Device (µPAD) for Determining Hydroquinone in Facial Whitening Cream using Phloroglucinol Reagent

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Abstract

A microfluidic paper-based analytical device (µPAD) is developed in this work to analyze hydroquinone in facial whitening creams using phloroglucinol. The µPAD features a hydrophobic barrier for detection and was fabricated using a wax printer with Whatman chromatographic paper. Detection was achieved by colorimetry based on the formation of an orange hydroquinone–phloroglucinol complex. The colored reaction product formed on the detection zone of the µPAD was scanned, and the images obtained were processed with Image-J software to determine their color intensity (RGB value). Optimization of the process conditions was conducted to achieve sensitive measurements. The optimum conditions yielding maximum sensitivity included a reagent addition sequence of phloroglucinol → NaOH → sample (hydroquinone), 1 µL of 0.5% phloroglucinol, 1 M NaOH, and 10-minute reaction. Under optimal conditions, the µPAD produced two linear calibration curves for hydroquinone at concentrations of 10–100 mg/L (R^2 = 0.9979) and 250–1000 mg/L (R^2 = 0.9991). The method demonstrated very good selectivity for the target analyte in the presence of propylene glycol and resorcinol with satisfactory validity and average recovery close to 100%. The proposed µPAD is a very simple and inexpensive technique for hydroquinone analysis and could be applied to cosmetics samples with satisfactory results.

Keywords: colorimetry, hydroquinone, image-j, phloroglucinol, µPAD

Introduction

Hydroquinone is a common active ingredient of whitening creams that acts as a depigmentation agent for the skin. It inhibits tyrosinase activity, which synthesizes melanin in the epidermis. Thus, in the presence of hydroquinone, skin pigmentation is inhibited and melanin production is reduced, resulting to a lighter complexion [1]. However, the use of hydroquinone in cosmetics, especially skin whitening creams, was banned in 2001 because the substance exerts harmful effects on the skin, including irritation, redness (erythema), and burning. The prolonged used of hydroquinone-containing cosmetics can cause leukoderma, exogenous ocronosis, and nephrotoxicity. Moreover, the overuse of hydroquinone-whitening cosmetics can lead to an overall change in the health status of the user, mutations, and even cancer [2, 3].

Hydroquinone determination in cosmetics can be achieved via several methods, including redox titration, thin-layer chromatography [1], spectrophotometry [4, 5], flow-injection spectrophotometry [6-8], and high-performance liquid chromatography (HPLC) [9,10]. These methods, especially the latter, are well known to offer accurate measurements and high precision and effectiveness. However, these methods also require a skilled operator and are not portable; thus, they cannot be used for on-site measurements.

Microfluidic paper-based analytical devices (µPADs) are a simple and inexpensive means for onsite analysis. This instrument-free technique is widely used in several countries as an efficient and effective analytical tool for qualitative and quantitative measurements. The µPAD design is based on the formation of a hydrophilic reaction zone with a hydrophobic barrier to control dispersion on a paper platform. The hydrophilic–hydrophobic barrier produces channels that limit the travel of a sample via capillary forces in the paper substrate and, thus, requires no external support [11]. The main detection modes for determining the concentration of an analyte include electro-

chemistry [12–14], colorimetry [15, 16], and electrochemiluminescence [17, 18]. Given their low cost and portability, μPADs have been developed for environmental analysis [19–21], biochemical analysis, and bioactive compound and disease detection [22–29]. However, a μPAD-based method for detecting hydroquinone in whitening cosmetics has yet to be reported.

Colorimetry is a very practical detection method because it requires only the naked eye for semi-quantitative measurements. For quantitative measurements, μPAD images may be captured using a digital camera or scanner and then processed using a personal computer (PC). The present research aims to develop a μPAD for detecting hydroquinone in whitening creams with phloroglucinol as a selective chromogenic reagent. Phloroglucinol has demonstrated good sensitivity, accuracy, and precision for spectrophotometric hydroquinone analysis [4–8]. In this study, hydroquinone is analyzed on the basis of the formation of an orange hydroquinone–phloroglucinol complex on μPAD paper under alkaline conditions. The hydroquinone concentration of a sample can be determined by measuring the color intensity of μPAD images obtained via digital printing techniques by using Image-J software. The process conditions of this new technique are also optimized to achieve the best performance for hydroquinone analysis.

Materials and Methods

Materials and Equipment. The equipment used in this work included a wax printer (Xerox ColorCube 8580 DN-2 type T2B047382) to print the hydrophobic barrier on Whatman No. 1 chromatography paper (CHR, Whatman, GE Healthcare Life Sciences, UK) for the μPAD. A hotplate was used to heat the wax ink barrier and allow it to penetrate through the paper to produce a flawless barrier for the reaction zone. A Canon PIXMA MP237 scanner and Image-J software were used to interpret the color intensity of captured images into red, green, and blue (RGB) values, which were then converted to an absorbance value by using the modified Lambert–Beer Law.

Hydroquinone (Sigma Aldrich, China) was used as the standard solution in all procedures from optimization to validation. Phloroglucinol and NaOH (99%) were purchased from Merck (Germany), and 95% ethanol was purchased from Sigma Aldrich. Phloroglucinol was used as a chromogenic reagent to form an orange complex with hydroquinone under alkaline conditions. Resorcinol and propylene glycol (Sigma Aldrich) were used for the selectivity test. Two types of whitening creams were used as real samples for method validation. All of the procedures employed in this work were referred from previous studies with some modifications [21, 29].

Device Preparation. CorelDraw X7 Graphics Suite software was used to design the μPAD, as shown in Figure 1. The μPAD consisted of a hydrophilic reaction zone (i.e., a circle with an inner diameter of 5 mm) and a hydrophobic barrier (thickness, 0.8 mm). The hydrophilic reaction zone enabled the reaction of the sample with phloroglucinol, while the hydrophobic barrier controlled dispersion and prevented leakage from the reaction zone. The μPAD was prepared by printing the fabricated design onto Whatman No. 1 chromatography paper (200 mm × 200 mm) using the wax printer with hydrophobic wax ink made of bisamide and maleic anhydride waxes. The printed devices were placed on a hot plate to allow the hydrophobic barrier wax to permeate through the paper, thus forming a completely hydrophobic barrier that could control liquid flow. The μPAD heating conditions were referred from the research of Wisang (2019) and Fauziah (2019) [29]. Here, the μPAD was covered with aluminum foil and heated at 120 °C for 90 seconds.

Method Optimization. The process conditions of the proposed μPAD method were optimized to enable sensitive measurements.

First, the proper sequence of reagent immobilization on the μPAD was determined. In this experiment, the order in which reagents were dropped onto the reaction zone was varied as follows: (A1) NaOH → phloroglucinol → hydroquinone and (A2) phloroglucinol → NaOH → hydroquinone. The sequence yielding the strongest color intensity was selected as the optimum addition sequence and used for the next experiment.

Next, the precise volume of phloroglucinol that could occupy the μPAD reaction zone was determined. Phloroglucinol volumes of 0.4, 0.6, 0.8, 1.0, and 1.2 μL were loaded onto the μPAD detection zone. The phloroglucinol volume that remained precisely within the detection zone was selected for further experiments.

Optimization was continued by varying the phloroglucinol (0.01%–1%) and NaOH (0.1, 0.5, 1.0, 1.5, and 2 M) concentrations to obtain the optimum values required to achieve the greatest intensity of the colored hydroquinone–phloroglucinol complex. The optimum concentrations of phloroglucinol and NaOH were then used for the following experiments.

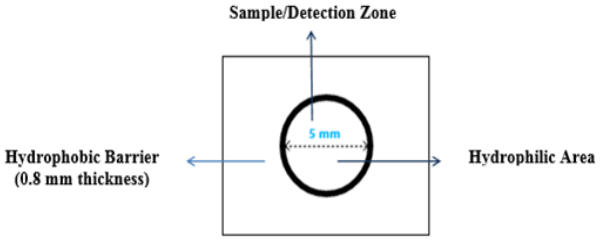


Figure 1. The μPAD Design for Hydroquinone Analysis

Finally, the reaction time was optimized to determine the minimum time for complex formation leading to the highest intensity and most evenly distributed color of the

reaction product on the detection zone. The reaction times were set to 10, 20, 30, 40, and 50 minutes. All optimum conditions determined were used for subsequent experiments.

Hydroquinone Determination. Hydroquinone detection under the optimum conditions determined in Section 2.3 was performed according to Figure 2. In this scheme, 1 µL of 0.5% phloroglucinol was dropped onto the µPAD detection zone. The device was allowed to stand for 5 min, and then 1 µL of 1 M NaOH solution was added to the detection zone. The device was allowed to stand for another 10 minutes to dry, after which it was considered ready for use. Hydroquinone detection could be achieved simply by dropping 1 µL of the sample onto the reaction zone of the µPAD device, allowing the paper to stand for 10 minutes, and then scanning the orange reaction product with a Canon PIXMA MP273 scanner. The color intensity of the images obtained were processed into RGB values by using Image-J software and then converted into absorbance values. The concentration of hydroquinone was determined by matching the absorbance obtained to a standard calibration curve.

Exactly 1 µL of various concentrations of standard hydroquinone (0, 10, 25, 50, 75, 100, 250, 500, 750 and 1000 mg/L) was dropped onto the µPAD, and processing was conducted as shown in Figure 2. The calibration curve was constructed by plotting the absorbance obtained as a function of the hydroquinone concentration added.

Method Selectivity. The selectivity of the µPAD method toward hydroquinone in facial whitening creams was determined by obtaining hydroquinone measurements with and without resorcinol and propylene glycol as interfering compounds. Various concentrations of resorcinol (0, 25, 50, 125, and 250 mg/L) were added to five

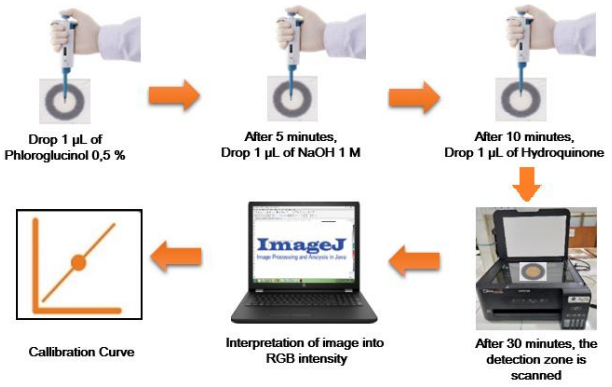


Figure 2. Operating Step of µPAD for Determining Hydroquinone

10 mL volumetric flasks containing 25 mg/L hydroquinone and diluted to the mark. The color intensity of these solutions was measured using the same procedure for hydroquinone determination (Figure 2), the corresponding RGB values were converted to absorbance, and the hydroquinone recovery was calculated. The same procedure was repeated for propylene glycol with the same various concentrations as resorcinol. The difference in hydroquinone concentration obtained between solutions with and without the interfering compounds was used to calculate the % error.

Method Validation. Method validation was achieved by using the µPAD to determine hydroquinone in two cosmetics samples via the standard addition technique. Exactly 0.10 g of whitening creams A and B were weighed out and gradually dissolved with distilled water in a 50 mL beaker glass. The solution was passed through fine filter paper, and the filtrate was filtered once more using a syringe filter. The filtered solution was transferred to a 100 mL volumetric flask and added with distilled water up to the mark. Afterward, the sample was diluted to obtain a concentration that is within the range of the calibration curve.

The concentration of hydroquinone in the samples was determined using the steps outlined in Section 2.4. Then, these procedures were repeated following the addition of 10 and 20 mg/L hydroquinone standard solutions to the samples. The percent recovery of hydroquinone in the samples was calculated by comparing the hydroquinone concentration recovered in the samples after standard addition with the actual hydroquinone concentration in the cosmetics.

Results and Discussion

The color in the reaction zone of the µPAD was produced by the reaction of phloroglucinol ions with hydroquinone under alkaline conditions. The color images obtained were analyzed using Image-J software to obtain RGB values, which were then converted to absorbance values by using the modified Lambert–Beer Law.

The principle behind the µPAD method for hydroquinone analysis is based on formation of an orange product in the detection zone via the formation of a hydroquinone–phloroglucinol complex. The reaction begins when 2 molecules of NaOH attract two hydrogen atoms from a phloroglucinol molecule to form a phloroglucinate ion (enolate; Figure 3), which acts as a nucleophile. Then, undergoes nucleophilic addition, during which a phloroglucinate ion attacks the C=C double bond of hydroquinone followed by electron delocalization and a phloroglucinol–hydroquinone complex may then occur through oxygen connections. Another mechanism through which the hydroquinone–phloroglucinol complex may be formed involves oxidative coupling. The

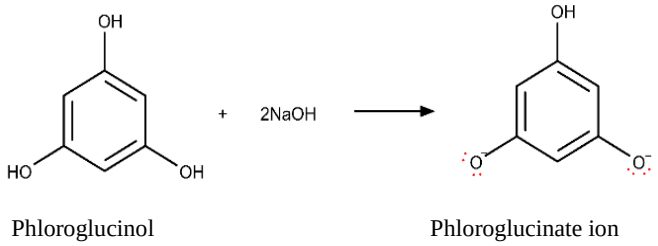


Figure 3. The Phloroglucinol Reaction under Alkaline Conditions

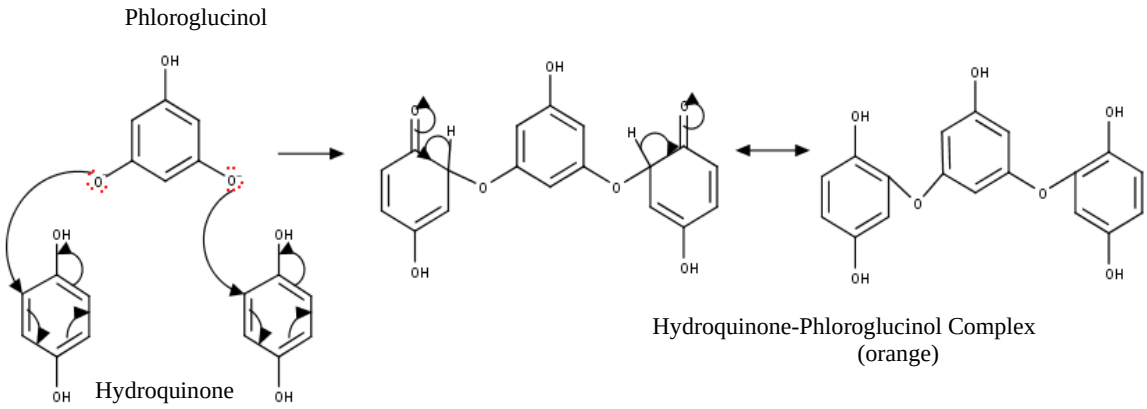


Figure 4. Estimated Reaction for the Formation of Hydroquinone-phloroglucinol Complex

oxidative coupling reaction refers to the joining of two molecules to form a C–O or C–C bond via an oxidation reaction. Hydroquinone and phloroglucinol are phenolic compounds that could be heterocoupled through C–O bonds. A possible reaction mechanism for the formation of the hydroquinone–phloroglucinol complex is depicted in Figure 4.

Determination of Optimum Conditions

Optimization of the Reagent Addition Sequence. The order in which reagents are dropped on to the μPAD may influence the formation of the phloroglucinol–hydroquinone complex and the sensitivity of hydroquinone measurement. Figure 5 shows that sequence A2 yields a more intense color in the μPAD detection zone than sequence A1. This finding may be explained by the extensive transformation of phloroglucinol into phloroglucinate ions, which act as nucleophilic groups facilitating the formation of the desired complex, promoted by sequence A2. As shown in Figure 5, the intensity of blue readings was much higher compared with those of red and green readings. Besides, the blue readings were linearly correlated with the color intensity (or absorbance) and hydroquinone concentration. This finding agrees with the results of Kohl [30], who found that a linear relationship between intensity and concentration may be achieved by using complementary color readings. Thus, blue readings were selected to measure the color

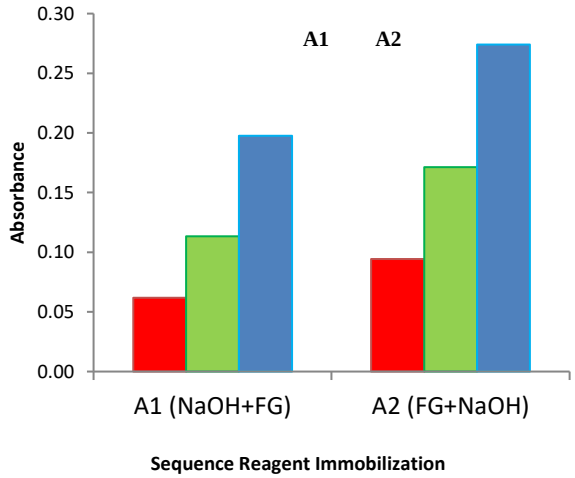


Figure 5. The Optimization of Sequence Reagent Immobilization

intensity of the μPAD images in subsequent experiments.

Optimization of the Phloroglucinol Volume. The optimum phloroglucinol volume could produce the highest color intensity of the phloroglucinol–hydroquinone complex precisely in the area of detection zone. The greater the phloroglucinol volume, the higher was the

color intensity (absorbance) of the complex, as shown in Figure 6. The absorbance determined using blue readings increased with increasing phloroglucinol volume up to 1 µL; however, phloroglucinol volumes exceeding 1.2 µL caused the complex to cross the hydrophobic barrier, which could lead to erroneous results. Therefore, a phloroglucinol volume of 1 µL was used for further optimization.

Optimization of the Phloroglucinol Concentration. The absorbance of the orange complex of phloroglucinol–hydroquinone increased initially with the phloroglucinol concentration up to 0.5% and then levelled off because all the hydroquinone has completely formed a phloroglucinol–hydroquinone complex (Figure 7). Therefore, 0.5% was considered as the optimal phloroglucinol concentration.

Optimization of the NaOH Concentration. The optimum NaOH concentration provides a suitable alkaline atmosphere for the formation of negatively charged phloroglucinol ions. The hydroxyl (OH^-) group of NaOH can attack the hydrogen in the OH^- group of phloroglucinol to form a phloroglucinate ion, which, in turn, could attack hydroquinone to form a heterocoupled phloroglucinol–hydroquinone complex. Figure 8 reveals

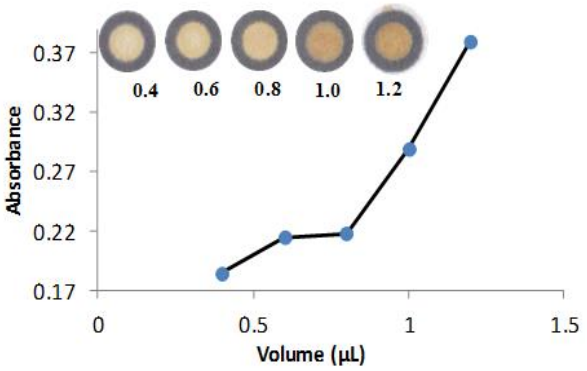


Figure 6. The Optimization of Reagent Volume

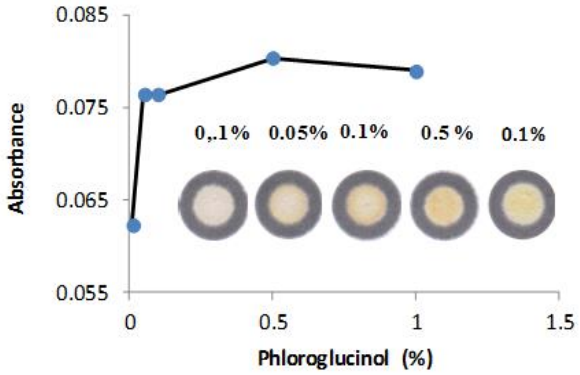


Figure 7. The Optimization of Phloroglucinol Concentration

that higher NaOH concentrations increase the color intensity of the µPAD images. The highest absorbance was obtained at a NaOH concentration of 1 M. Thus, 1 M NaOH was used for subsequent experiments.

Optimization of the Reaction Time. The reaction time was optimized to determine the shortest scanning time and avoid the color degradation of the complex compounds. A short reaction time can result in the incomplete formation of the phloroglucinol–hydroquinone complex. However, long reaction times may degrade the complex color via exposure to light and unsuitable temperature and pH. A reaction time of 10 minutes yielded optimum results with maximum absorbance (Figure 9). This reaction time was used for subsequent experiments.

Standard Curve and Linearity Measurements. Under the optimum conditions obtained above (i.e., A2 reagent immobilization sequence, 1 µL of 0.5% phloroglucinol, 1 M NaOH, and 10-minute reaction), the µPAD method by using 1 µL of sample demonstrated clear differences in color intensity as the hydroquinone concentration was varied from 10 mgL^{-1} to 1000 mg/L (Figure 10). When the RGB values of the color intensity of the images obtained were converted to absorbance values and the latter was plotted as a function of hydroquinone concentration, very good correlations (i.e., R^2 close to 1) were

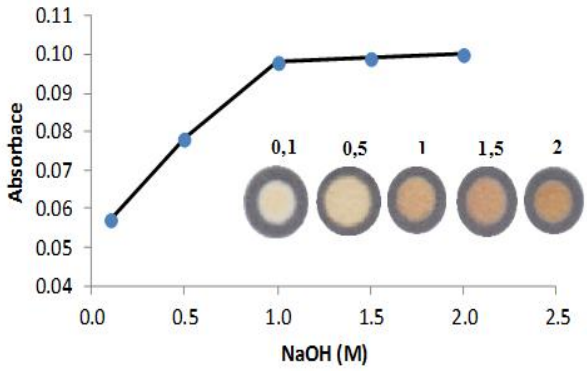


Figure 8. The Optimization of NaOH Concentration

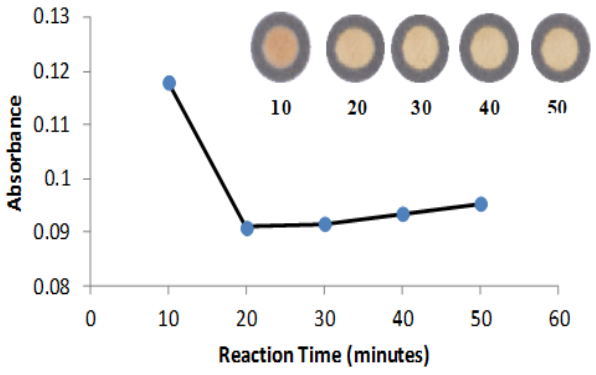


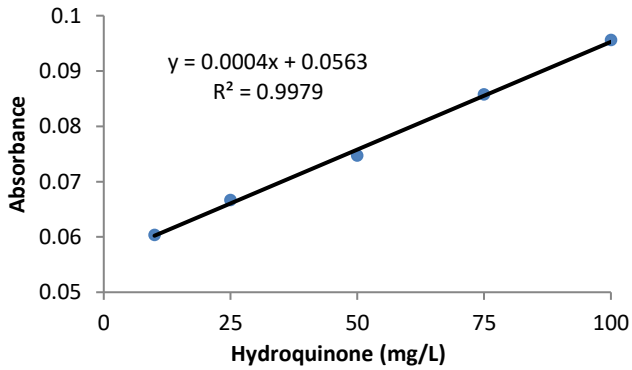
Figure 9. The Optimization of Reaction Time

obtained over the concentration ranges of 10–100 mg/L (Figure 11-a) and 250–1000 mg/L hydroquinone (Figure 11-b). The μPAD images presented colors with greater intensity under high hydroquinone concentrations and lower intensity under low hydroquinone concentrations. In other words, the higher the hydroquinone concentration, the greater was the color intensity of the orange phloroglucinol–hydroquinone complex.

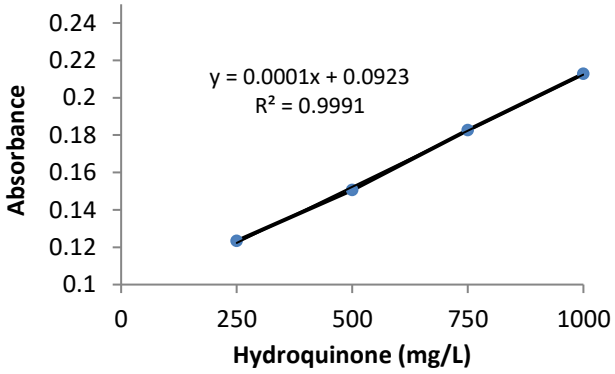
According to Figure 11, the hydroquinone concentration is proportional to the color intensity of the μPAD image; specifically, the greater the hydroquinone concentration, the higher the absorbance value obtained from the intensity of the blue readings. The standard curve for hydroquinone at concentrations ranging from 10 mgL^{-1} to 100 mgL^{-1} provided a linear regression equation of y = 0.0004x + 0.0563 (R^2 = 0.9979). Similarly, the relationship between hydroquinone concentration and absorbance gave a linear regression equation of y = 0.0001x + 0.0923 (R^2 = 0.9991) at hydroquinone concentrations of 250–1000 mgL^{-1}. In this work, R^2 values close to 1 indicate very good linear correlations between concentration and absorbance.

Method Selectivity. The selectivity of the μPAD method was investigated by adding separately resorcinol and propylene glycol, two substances that are commonly present in whitening cosmetics, to a standard hydroquinone solution. As depicted in Table 1, addition of resorcinol at concentrations of 25, 50, and 125 mg/L did not significantly affect hydroquinone measurements obtained using the μPAD method. This finding is supported by the small % error generated (<10%). Hydroquinone measurements obtained following the addition of 250 mg/L resorcinol (1:10) showed a slight increase, with a % error of 10.82%. The results of a *t* test at the 95% confidence level showed that t_{count} (3.65) is greater than t_{table} (2.92). Thus, addition of resorcinol to a sample at amounts of 10 times greater than the hydroquinone concentration can increase the measured concentration of the latter. Addition of propylene glycol at concentrations of 25, 50, 125, and 250 mg/L did not interfere with the measurement of hydroquinone concentration, as indicated by the low % error determined from the experiments.

Figure 10. The results of μPAD color intensity on the determination of the standard curve. Condition: 10-1000 mg/L hydroquinone



(a)



(b)

Figure 11. Two Linear hydroquinone standard curves 11-a: Linear from 10-100 mg/L; 11-b: Linear from 250-1000 mg/L

Table 1. Selectivity of μPAD Method

Hydroquinone (mg/L)	Interfering Compound	Interfering compound (mg/L)	Hydroquinone measured ± SD (mg/L)	% Error
25	Resorcinol	0	25.39 ± 4.27	1.58
		25	25.61 ± 2.00	2.46
		50	25.73 ± 7.31	2.93
		125	27.38 ± 29.02	9.53
		250	27.70 ± 1.19	10.82
25	Propylene Glycol	0	25.39 ± 4,27	0,73
		25	26.91 ± 1,53	7,64
		50	26.58 ± 6,65	6,33
		125	26.04 ± 11,08	4,17

	250	26.89 ± 5,26	7,54

Table 2. Validation of µPAD Method

Sample	[HQ] added (mg/L)	[HQ] measured (mg/L± SD)	% Recovery
A	0	27.24 ± 0.11	-
	10	37.16 ± 4.65	99.72
	20	47.46 ± 3.30	100.81
B	0	28.22 ± 2.03	-
	10	38.55 ± 0.95	101.17
	20	48.22 ± 5.04	100.01

Method Validation. The validity of the µPAD method was assessed by detecting hydroquinone in two types of whitening cream cosmetics. The results of the validation test are presented in Table 2. The µPAD method showed very good accuracy and validity, as supported by recovery values in the range of 95%–105%, as well as high precision (%RSD < 10%).

In summary, the µPAD method proposed in this work provides satisfactory accuracy and precision. Therefore, the fabricated device can be used as an alternative method for detecting hydroquinone in whitening cream cosmetics.

Conclusion

Hydroquinone in whitening creams can be determined using the proposed µPAD, which is based on the simple reaction of hydroquinone with phloroglucinol under alkaline conditions to form an orange hydroquinone–phloroglucinol complex. This method could be used to determine hydroquinone concentrations in the ranges of 10–100 and 250–1000 mg/L. Although the µPAD developed in this work is less sensitive compared with other advanced methods, it involves a simple process and is inexpensive. The proposed µPAD device may be used as a test kit for monitoring hydroquinone in facial whitening creams with fairly high accuracy and precision.

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