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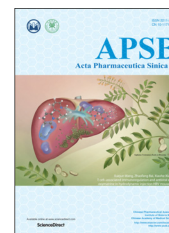

SHORT COMMUNICATION

Scanometry as microplate reader for high throughput method based on DPPH dry reagent for antioxidant assay

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KEY WORDS

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Abstract The stable chromogenic radical 1,1′-diphenyl-2-picrylhydrazyl (DPPH) solution was immobilized on the microwell plate as dry reagent to construct a simple antioxidant sensor. Then, a regular flatbed scanner was used as microplate reader to obtain analytical parameters for antioxidant assay using one-shot optical sensors as scanometry technique. Variables affecting the acquisition of the images were optimized and the analytical parameters are obtained from an area of the sensing zone inside microwell using the average luminosity of the sensing zone captured as the mean of red, green, and blue (RGB) value using ImageJ® program. By using this RGB value as sensor response, it is possible to determine antioxidant capacity in the range 1–25 ppm as gallic acid equivalent (GAE) with the response time of 9 min. The reproducibility of sensor was good (RSD < 1%) with recovery at 93%–96%. The antioxidant sensor was applied to the plant extracts, such as sappan wood and Turmeric Rhizome. The results are good when compared to the same procedure using a UV/Vis spectrophotometer.

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1. Introduction

Antioxidant capacity is a broadly used term as a parameter to characterize different substances and food samples with the ability of scavenging or neutralizing free radicals. This capacity is associated to the presence of compounds capable of protecting a biological system against harmful oxidation[1]. There are several synthetic radicals, such as 2,2′-azinobis-3-ethylbenzothiazoline-6-sulfonic acid (ABTS), 1,1′-diphenyl-2-picrylhydrazyl (DPPH), and *N*,*N*-dimethyl-*p*-phenylendiamine (DMPD), employed for determining antioxidant capacity of various samples[2–5]. The DPPH method is one of the most frequently used to assess the ability of compounds as free radical scavengers or hydrogen donors, and to evaluate the antioxidant capacity of food samples. The method was introduced by Blois[5], and improved by some authors for measuring antioxidant activity of numerous substances and determining antioxidant capacity of various food and plant samples[3,6,7]. In brief, this method is based on the reduction of the chromogenic DPPH radical by an antioxidant, such as in a plant extract, which causes the radical to change color, and this change can be monitored and quantified using spectrophotometer at 515–520 nm[8]. The radical DPPH is stable and does not have to be generated for hours before the analysis, as in other radical scavenging assays[9].

In conventional spectrophotometric method, a large volume (1.0–5.0 mL) of freshly prepared DPPH solution in a cuvette is required[10–15]. In order to reduce large amount of DPPH solution, a microwell plate can be used in the assay, as it was done by Lee et al.[16]. Lately, it was known that no significant different parameters (repeatability, reproducibility, percentage recovery) were observed between microwell and cuvette-based method within intra-laboratory validation[9]. The DPPH microwell-based method is continuously used then by some authors as high throughput screening for antioxidant capacity[17–20]. To make the assay simpler and faster as high throughput screening, the microwell was used as solid support for DPPH in dry reagent format as described elsewhere[19]. By adding methanol or ethanol into the wells, the system can be used for high throughput antioxidant screening of various samples (banana, green tea, pink guava, and honey dew), and it was shown that the results were in good agreement with that of conventional DPPH-microwell platform. However, this method is not suitable for field analysis since specialized, cost-expensive instrument such as microplate reader (spectrophotometer) is required to conduct the assay. To overcome this limitation, flatbed scanner can be used as a microplate reader to obtain digital color image which can be further analyzed quantitatively.

Recently, using scanner as scanometric technique gained its popularity due to its application in various chemical and biochemical assays. The scanometric technique which relies on either a light scattering instrument or flatbed scanner coupled with various probes or sensors can be used for the detection of bacteria, dopamine, magnesium ions, lead ions, thrombin, and mercury ions[21–26]. Being subclass of colorimetry, scanometry uses a gray scale as opposed to the various color space. The gray intensity, typically a result of silver enhancement is the measured signal in scanometry[27]. Scanometry was used to characterize optical feature of various dyes, such as disperse orange 3, methyl orange, fluorescein, eosin Y, rhodamine B, trypan blue, prussian blue, malachite green, methylene blue, chlorophyll b, and DPPH, in a microwell plate[28]. As the color intensity in red, green, and blue (RGB) value was mathematically converted to RGB-resolved absorbance, it was shown that flatbed scanner was comparable with spectrophotometer.

Here, we propose a scanometric technique for conducting DPPH assay. In this work, DPPH solution was immobilized on a 96 microwell as dry reagent to construct antioxidant sensor. Then, a regular flatbed scanner was used as microplate reader to evaluate antioxidant capacity of several plant extracts. When it was compared with other DPPH-based sensors[29,30], the proposed sensor is simpler, since in other optical sensors the DPPH solution has to be immobilized in polymer (e.g., PVA, PVC) in a long time chemical synthetic reaction to construct the sensor. Moreover, the sensors have to be transferred into a cuvette prior to antioxidant assay in UV/Vis spectrophotometer which made the afore mentioned methods[29,30] need longer procedure than the proposed method. As regular flatbed scanner was employed for obtaining sensor response, the developed method is extremely cheaper than microplate reader (ELISA reader) or UV/Vis spectrophotometer. In the microplate reader, the absorbance of DPPH after antioxidant addition was measured, while in the proposed scanometric technique, the color intensity (mean RGB) of DPPH after antioxidant addition has been measured for determining antioxidant capacity. Hence, it is obvious that, using scanometric technique, no need the samples to be transparence, as in our previous work[19]. In this paper, even non-transparence or opaque sample can be used, since the different analytical response and different instrument were used as a reader for antioxidant sensor response. In addition, we also used less reagent concentration (125 µg/L) compared to the previous one (150 µg/mL)[19]. Thus, it make reagent used more efficient, as it is used in one shot measurement. Furthermore, the developed method can be suitable for field analysis and/or in remote area, where medicinal plant extracts can be screened for their antioxidant capacity on site.

2. Materials and methods

2.1. Chemicals

Gallic acid (GA) and DPPH were obtained from Sigma–Aldrich (USA). Methanol was purchased from Merck (Germany). All chemicals were of analytical reagent grade.

2.2. Herbal samples

Herbal samples used in this work, *i.e.*, Sappan wood (*Caesalpinia sappan* L.), and Turmeric Rhizome (*Curcuma domestica* Val.), were purchased from local market of Jember, East Java. All herbal samples were authenticated and deposited at Pharmacognosy Laboratory, Faculty of Pharmacy, University of Jember, Indonesia. Herbal samples were air dried and powdered until their particle size freely passed through sieve 100 mesh.

2.3. Sensor fabrication

Sensor fabrication was done as in our previous work[19] with slight modification. A solution of DPPH in methanol at various concentrations (50, 100, 125, and 150 ppm) were transferred (200 µL) into 96 microwell plate as matrix sensor. The solvent

was then evaporated under mild condition at room temperature to construct antioxidant sensor based on DPPH. Afterward, the antioxidant sensor was ready to be used. For long term used and avoid photodecomposition of DPPH, the antioxidant sensor was sealed with aluminum foil.

2.4. *Extraction of herbal samples*

All herbal powder were extracted using method reported by Ningsih et al.[31] with slight modification. The dried herbal powder (10 g) was extracted with methanol (300 mL) for an hour at 30 °C using an ultrasonicator bath (Elmasonic S180H, Germany). The extracts were then filtered through Whatman filter paper No. 1 (USA) by using vacuum funnel. Afterward, the filtrates were separated and stored in well-capped tubes prior to antioxidant assay at room temperature.

2.5. *Optimization study*

In order to find the optimum DPPH concentration that give best calibration curve in term of linear correlation and the slope, GA was used as standard solution, since it is classified as intermediate based on its antioxidant kinetic, which is more suitable for the optimization study than that of antioxidants with rapid kinetic (ascorbic acid) or slow kinetic (ferulic acid)[7]. GA solution was added in different concentration (1, 5, 10, 15, 20, and 25 ppm) to each well 150 µL of methanol and 50 µL of GA solution were added. After 9 min, the color change of each well was quantified using the measurement procedure, where the calibration curve can be constructed.

2.6. *Measurement procedure*

The color change of sensor was captured using flatbed scanner (Canoscan, LIDE 110, Japan) in which color photo mode with resolution at 300 dpi was set for image scanning. The color intensity was then analyzed with ImageJ® program for Windows®. The color intensity of sensors (ΔRGB) was obtained by subtracting the intensity value of mean RGB of the application of sample from the intensity value of mean RGB without the sample. All of the experiments were carried out in triplicate measurements.

3. Results and discussion

3.1. *The antioxidant sensor*

The fabrication of antioxidant sensor based on DPPH was performed by surface coating on the bottom of the 96 microwell plate with the 200 µL DPPH solution (125 ppm) as it can be seen in our previous work[19]. The process was very simple, by just evaporate the DPPH solvent in 40 min, then leaving DPPH dry reagent as immobilized reagent on the bottom of each well. After this immobilization process, the antioxidant sensor was ready to be used as antioxidant sensor.

3.2. *Sensing scheme*

In this sensing scheme, DPPH as radicals is used as the basis for optical detection in the assessment of antioxidant activity[5–7]. This is due to the fact that DPPH has been widely used for the determination of antioxidant activity of phytochemicals, such as flavonoids and polyphenols[18,19]. In this assay, the purple of chromogen radical ($DPPH^{\bullet}$) is reduced by antioxidant (*e.g.*, GA) to the corresponding pale yellow hydrazine (DPPH-H). Reduction of the chromogenic purple radical ($DPPH^{\bullet}$) by hydrogen-donating antioxidant is monitored by capturing its color change to the pale yellow of the antioxidant sensor. The sensing mechanism is shown in Fig. 1, where AH is donor molecule, and $A^{\bullet}$ is free radical produced.

This DPPH as chromogenic radical reagent was chosen, since it is widely used in well-established procedures for the antioxidants assay, together with sufficient chemical stability in a dry reagent format, avoiding problems associated with leaching[19], as it will dissolve again when sample introduced. In addition, there is no need for regeneration of radicals *in situ*, which makes the antioxidant sensor simpler and more practical.

3.3. *Optimization of DPPH concentration*

The antioxidant sensor works as the color change from purple to yellow by introducing antioxidant such as GA; therefore, the optimum color change of the sensor would be depend on the DPPH concentration. In order to optimize the DPPH concentration in term of its calibration curve, various concentration of DPPH have been immobilized as antioxidant sensor and tested toward the increased concentration of GA (1–25 ppm). After scanometric measurements, the calibration curves of GA *vs*. color intensity (ΔRGB) were made for each DPPH concentration (50, 100, 125 and 150 ppm) tested as given in Table 1. The antioxidant sensor with 125 ppm DPPH was selected as optimum DPPH concentration in term of its linear correlation and slope. The slope and coefficient correlations (r) of the 125 ppm DPPH have been found to

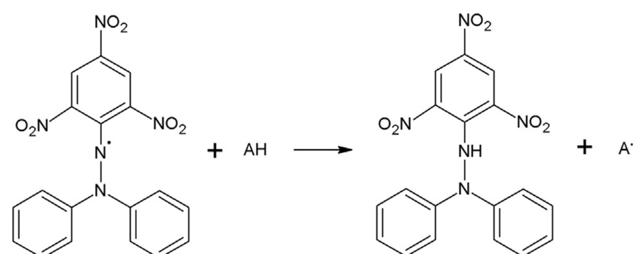

Figure 1 The reaction mechanism of DPPH radical with antioxidant (AH).

Table 1 The calibration curves of gallic acid (1, 5, 10, 15, 20, and 25 ppm) towards various DPPH concentrations for the antioxidant sensor ($n=3$).

DPPH conc. (ppm)	r^2	Equation of calibration curve	Slope
50	0.9873	$y=3.2809x+0.4224$	3.2809
100	0.9911	$y=3.3118x-1.1945$	3.3118
125	0.9922	$y=3.4257x-3.3301$	3.4257
150	0.9912	$y=3.2353x-1.3858$	3.2353

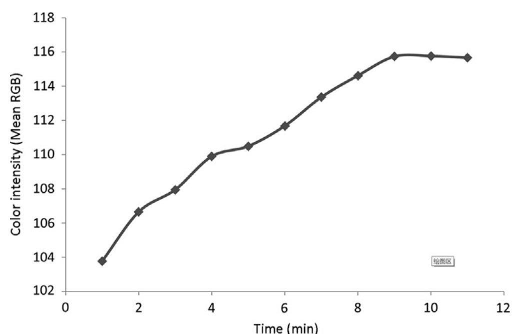

Figure 2 Response time of the antioxidant sensor towards gallic acid (15 ppm).

Table 2 The antioxidant sensor response towards 10 ppm of gallic acid ($n=6$).

Sample	RGB	ΔRGB
Blank	43.62	–
1	71.724	28.104
2	70.814	27.194
3	71.237	27.617
4	71.603	27.983
5	71.712	28.092
6	71.301	27.681
Mean		27.779
RSD (%)		1.271

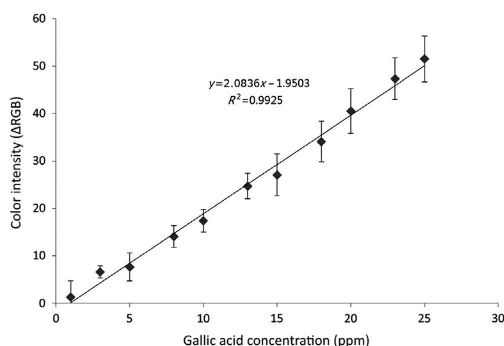

Figure 3 Sensor response towards gallic acid (1–28 ppm), $n=3$.

be 3.426 and 0.996, respectively, which is higher compared to other DPPH concentrations (50, 100 and 150 ppm) as it can be seen in Table 1. Therefore, the antioxidant sensor with DPPH concentration of 125 ppm was used for further measurements.

3.4. Response time

The response time of antioxidant sensor was investigated using 15 ppm GA solution. The response of sensor was recorded at every 2 min until stable color intensity value was obtained. The response time of sensor was observed at 9 min. After this period, the sensor gave stable response as shown in Fig. 2. Therefore, this response times was used for further measurements.

It can be seen in Fig. 2 that the developed sensor has response time at 9 min which is faster than that of other DPPH-based optical sensor[30], while the similar GA concentration was applied (±1 mmol/L). Moreover, our sensor takes a short operating time in the term of time of analysis, while DPPH absorbance was typically read at 30 min after addition of tested samples in other microwell-based methods[18,19]. Similar time of analysis (10 min) was also reported with observed in other microwell-based method[20]; however, the method was suffered from co-solvent and/or buffer addition. Hence, it can be drawn that our developed method is simpler and faster than afore mentioned methods.

3.5. Antioxidant determination

GA was used as standard for antioxidant assay in this work. The calibration curve was constructed by plotting concentration of GA *vs*. sensor response as ΔRGB value as depicted in Fig. 3. It can be seen that the linear range of the sensor response is in the range of 1–25 ppm with the coefficient correlation (r) of 0.996. The detection limit (LOD) of the antioxidant sensor, which is defined as the concentration of sample yielding a signal equal to the blank signal plus three times of its standard deviation[32], was calculated to be 0.762 ppm. The reproducibility of the sensor response was tested toward 10 ppm of GA solution as given in Table 2, the precision of sensor can be observed. It can be seen that RSD value was lower than 2%, indicating the developed method has good precision[32].

3.6. Recovery

The percentage recovery values for the accuracy of the sensor on the determination of antioxidant activity are performed by adding 30%, 45% and 60% to initial 12, 13, and 15 ppm of GA in sample simulation. The mean of percentage recovery (%) was calculated to be 91%–96% as it can be seen in Table 3. This results were in good agreement with theoretical recovery values (%) for unit concentration of 10 ppm (80%–110%)[33]. Therefore, the developed method indicated a good accuracy for antioxidant measurements.

3.7. Sensor stability

In this work, various storage conditions were applied for stability test of the developed sensor. The sensor was stored separately in a well-capped cabinet at room (30 °C) and chiller (4 °C) temperature. Then, the sensor response towards GA (1–25 ppm) was

Table 3 Recovery study of gallic acid (%) in simulated sample using the antioxidant sensor ($n=3$).

Sample	Found concentration (ppm)	Recovery (%)
Initial (10 ppm)	10.135±0.008	101.350±0.084
30% addition	2.546±0.075	83.747±2.397
45% addition	4.417±0.063	96.840±1.462
60% addition	6.115±0.365	100.551±5.924

observed in every week, until 10% decrease of initial response was obtained. After two weeks, sensor response was observed to decrease more than 10% when sensor was stored at room temperature as it can be seen in Table 4. Hence, it can be noted that stability of sensor was only maintained during one week storage at room temperature. This finding seemed to be found in other DPPH-based optical sensor, in which the absorbance of DPPH-polymer film was completely loss within one week storage at room temperature[30].

Table 4 The decrease of sensor response towards gallic acid (1–25 ppm) after it was stored in room temperature (30 °C).

Gallic acid	Storage time (week)				
conc. (ppm)	1	2	3	4	5
1	9.16	12.93	15.45	17.22	19.14
5	2.19	11.02	13.34	13.50	14.59
13	7.34	12.68	13.89	15.83	44.21
25	3.46	12.53	11.85	12.02	15.02

In order to obtain the stable sensor response, the sensor was also stored in chiller temperature right after fabrication step as it was described earlier. Table 5 shows that sensor response towards GA (1–25 ppm) was found to be stable during six-week storage at chiller temperature. After six week, the sensor response was decreased more than 10% (data not shown), suggesting that stability of sensor was only preserved during six week storage at chiller temperature.

Table 5 The decrease of sensor response towards gallic acid (1–25 ppm) after it was stored in chiller temperature (4 °C).

Gallic acid	Storage time (week)					
conc. (ppm)	1	2	3	4	5	6
1	0.81	1.40	2.33	3.21	7.45	7.67
5	3.12	3.67	3.68	3.92	4.87	5.29
13	4.80	6.07	6.40	7.26	7.43	8.22
25	1.50	2.61	2.83	4.69	7.12	7.49

3.8. *Application on plant extracts*

In order to demonstrate the practical used of scannometry, various plant extract, such as sappan wood and Turmeric Rhizome, were carried out. Here, Sappan Lignum and Turmeric Rhizome were used with some purposes. Firstly, they were used to show that the color of herbal extracts did not affect the measurement of antioxidant capacity (the color of sappan extract is red, while the color of turmeric is yellow), and that the sensing mechanism relied on redox activity only. Secondly, the sample is taken based on the part of plant organ used for medication, *e.g.*, sappan is represented woods, while turmeric is represented rhizome. Thus, the proposed antioxidant sensor can be used for determination of antioxidant activity in any part of plant organ (leaf, root, flower, etc.) extracts or other plants extracts.

In this work, antioxidant capacity of plant extracts were compared with that of GA, as this approach was also reported in some literatures[34–37]. Using calibration curve of GA (5–25 ppm) made by the scanometric technique, the antioxidant capacity of extract was calculated, as it was expressed as gallic acid equivalent in ppm (ppm GAE). As comparison, UV/Vis spectrophorometry was used for the assay of antioxidant capacity of sample plant extracts with absorbance reading at 515 nm. Then, the results based on the scanometry were compared with the assay using UV/Vis spectrophotometer. The results showed that the proposed method was in good agreement with the spectrophotometric method, as it can be seen in Table 6, indicating the feasibility of the proposed scanometry for the determination of antioxidant capacity of the plant extracts.

4. Conclusion

A scanometry has been used as a microplate reader for high throughput screening of antioxidant based on DPPH as dry reagent on microwell plate as also used by other[28]. However, the proposed method is the first application for antioxidant sensor, measuring antioxidant capacity of GA and plant extracts. In this work, the developed method has linear range at 1–28 ppm with LOD at 0.794 ppm, and it was found to be reproducible, and good recovery for antioxidant determination of several plant extracts. The used of scanner in this proposed method is simple, easy to

Table 6 The comparison results of antioxidant capacity (ppm GAE) of various plant extracts determined by the antioxidant sensor and the UV/Vis spectrophotometer ($n=3$, $\alpha=0.05$).

Sample extracts	Antioxidant sensor-scanometric	UV/Vis Spectrophotometer	Significance value (P)
Sappan wood (10%, *w*/*v*)	10.22 ± 0.07	10.55 ± 0.21	0.062
Turmeric Rhizome (0.1%, *w*/*v*)	10.72 ± 0.26	11.08 ± 0.09	0.082

operate, and low-cost, as well as it can be used as an alternative reader for the microwell plate.

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