Developing a Mickey-Mouse-Designed Microfluidic Paper-Based Analytical Device (μPAD) to Determine The Antioxidant Activity of Green Tea

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Abstract. The determination of the antioxidant activity in green tea using FRAP essays developed in a Microfluidic Paper-Based Analytical Device (μPADs) was observed. μPAD was prepared on the chromatographic paper with a suitable pattern and then printed using a solid ink printer. The solid printing was intended to obtain a hydrophobic barrier and a hydrophilic channel on the chromatographic paper. A preliminary study was done to determine the optimum time and temperature used on the penetration of obtaining the hydrophobic barrier to avoid leakage in the channel. Optimization of temperature and time was calculated by the average velocity, the optimum condition was obtained at 120 °C for 90 seconds with an average speed of 0.1 mm.s⁻¹. The green tea samples were prepared by extracting its active compound using demineralized water at 25 °C and 90 °C with 2 hours of immersion time. For the measurement of antioxidant activities, the analysis was carried out by placing 0.5 μl in the detection zone and 5 μl samples into the sample zone μPAD. Then, the color reaction product, which propagates from the sample zone to the detection zone, is processed by Image J software to measure color intensity in CMYK mode. Extracts of green tea samples at 25 °C and 90 °C has a significant difference in antioxidant activity. These results indicate that the method developed in this work can be used as an alternative method for analyzing antioxidant activity in green tea extract. The results of the average amount of antioxidant activity in green tea samples were shown with Fe²⁺ concentration. High-speed detection, low cost, high accuracy, and ease of use can be attributed to the advantages of our μPAD method.

Keywords: Microfluidic devices, antioxidant activity, phenanthroline and FRAP

1. Introduction
The increasing probability of getting infected by degenerative diseases can now be prevented as the research and technology developed. It may be prevented by consuming nutrition additive products contain several antioxidants, which mainly from natural resources. The antioxidant has an important role in maintaining a healthy body [1–4]. Moreover, the antioxidant is a molecule that is able to slow down or prevent the oxidation of other molecules [5] which is known as antioxidant activity.
Antioxidant activity can be determined through a redox-linked colorimetric method [6]. It is recognized by the color changing of colorimetric indicator as a result of the ferric reducing (from Fe$^{3+}$ to Fe$^{2+}$) ability of the antioxidant [6, 7]. The methods which have been developed to determine the antioxidant activity in many samples including spectrophotometry methods are DPPH, ABTS, CUPRAC, ORAC, and FRAP. FRAP method has been chosen for its simplicity, fast, accurate and sensitivity in plan-based antioxidant activity determination [1, 6].

Tea is the second most consumed beverage from the *Camellia sinensis* plant, contains antioxidants in which have health benefits [3, 8]. Green tea is one of the tea products which have various benefits for health due to its high flavonoid compound [2, 3]. Green tea has been known as an effective tea in preventing many diseases associated with aging, also as an anti-inflammatory, anti-proliferative and anti-atherosclerotic [2,3,8]. Antioxidant activity of green tea can also be determined using modern instruments through *high-performance liquid chromatography* (HPLC) [3,9]. The time-consumed preparation of samples, non-portable and relatively high-cost instrument are some factors which provide some disadvantages to the researchers using HPLC.

A paper-based microfluidic (µPAD) analysis method is developed to overcome the disadvantages of analytical methods in determining the antioxidant activity. The interesting thing in µPAD is the used of chromatographic paper as a substrate material for the movement of samples. The µPAD is known as an inexpensive, portable, easy to use, biocompatible and can be easily disposed of [10–12]. It is unnecessary to provide an external pump to enhance the capillary driven of the samples because µPAD is considered as self-priming capillary pump [10]. There is still limited study on using µPAD to determine the antioxidant activity of green tea by the redox-linked colorimetric method. Therefore, the objective of this study was to determine the antioxidant activity through a redox-linked colorimetric method using a mickey-mouse-designed microfluidic paper-based analytical device (µPAD).

2. **Materials and Method**

2.1 **Materials and Chemicals**

Whatman chromatographic paper no. 1 (Whatman TM, GE Healthcare, UK) was used as the chromatographic paper for the µPAD device. Xerox ColorCube 8580 DN-2 type T2B047382 printer (Xerox, US) and a Hotplate (Menmet) were used to obtain the hydrophobic barrier on the chromatographic paper. HP Deskjet 2130 scanning device and Image J software were used to obtain the level of the red, green and blue color of the color change occurred on the redox reaction between sample and Fe$^{2+}$ and phenanthroline.

Four different brands of green tea samples, used in the study, were purchased from a local supermarket. Ferric chloride hexahydrate (Sigma, Aldrich) was used as the reagent to determine the antioxidant activity, 1, 10 - phenanthroline (E Merck, Germany) were used to indicate the color changed.

2.2 **Preparation of µPAD**

The µPAD design was obtained using the CorelDraw X8 application with a 0.8 mm line thickness. The design (Figure 1) was printed on Whatman chromatographic paper No. 1 using Xerox ColorCube 8580 wax printer with CMYK installed ink. The hydrophobic barrier was then activated on a hotplate at 120 °C for 30, 60, 90 and 120 seconds. Capillary driven was observed at some time of penetration and then the fastest speed will be used in this study.
2.3 Sample and standard preparation
The green tea sample was prepared according to Kodama et al [2] study with some modifications. Four green tea products were used as the sample. 0.2 gram of green tea sample was immersed in two different temperatures of water, 25 °C and 90 °C, for 2 hours. The green tea extracts were then used as the sample for antioxidant activity determination.

To validate the result of the μPAD, a standard was prepared using gallic acid. The gallic acid was prepared according to Sirivibulkovit et al. [11] experiment with some modifications. The concentration of gallic acid standards used in the study were 25, 30, 55, 75, 80 and 100 mg L⁻¹.

2.4 Antioxidant activity determination of green tea
Antioxidant activity was determined using the μPAD method according to the study of Sateanchok [12] with several modifications. The determination was carried out through the FRAP method (Figure 2). The FRAP reagents, contain FeCl₃ and phenanthroline, were dropped in the detection zones. Then green tea extract samples were then immersed in the sample zone. The color changes that occurred were scanned and observed. The μPAD with the color changed results were undergone scanning process (using HP Deskjet 2130 scanning device). The scanning result images were then observed using Image J software to determine RGB values.

Figure 1. The μPAD design used in the study

Figure 2. The method to determine the antioxidant activity applied during the study. (a) The FRAP reagent FeCl₃ was placed at the detection zone along with the 1,10 – phenanthroline, (b) the green tea sample was placed in the sample zone and allowed to immerse to the detection zone through the channel, (c) the sample and the reagents were integrated and color changes were obtained. (d) The color change was then scanned and e. the scan result was observed in ImageJ software.
3. Result and discussion

3.1 Optimization of the μPAD fabrication

There are four methods/techniques for fabricating μPAD, such as photolithography [11], cutting [10,13], screen printing and wax printing [10,14]. Wax printing is a method that mostly chosen to fabricate a competitive hydrophobic barrier in mass printing [7]. The μPAD designs, obtained from the study, after penetration of wax ink at the temperatures of 100 °C, 110 °C, 120 °C and 130 °C for 30 s, 60 s, 90 s, and 120 s were presented in Figure 3. It was shown that the longer penetration time to penetrate the wax ink into the μPAD, the more solid/stable the hydrophobic barrier. The penetration time of 120 seconds generated the most solid hydrophobic barrier to the μPAD as compared to the μPAD penetrated with other penetration times.

**Figure 3.** The scanned μPAD design seen from front and back after the various penetration temperatures and times.
The penetrated solid ink µPADs were then validated for the capillary driven. The capillary driven of the penetrated µPAD was recorded in Table 1. Table 1 presented that the capillary driven of the µPADs were varied between the penetration times and penetration temperatures. Penetration temperature of 120 °C with penetration time of 90 seconds generated the fastest capillary driven of µPAD. Therefore, µPAD obtained from penetration temperature of 120 °C and penetration time 90 seconds was used for further study, which is the determination of antioxidant activity in green tea.

Table 1. Capillary drove of solid ink µPAD penetrated with different penetration temperature and penetration time

| Penetration Temperatures (°C) | Penetration Time (s) | Channel Length (mm) | Capillary Time (s) | Capillary Driven mm.s⁻¹ |
|-----------------------------|---------------------|---------------------|--------------------|-------------------------|
| 100                         | 30                  | 10                  | 183                | 0.05                    |
|                             | 60                  |                     | 166                | 0.06                    |
|                             | 90                  |                     | 145                | 0.06                    |
|                             | 120                 |                     | 122                | 0.08                    |
| 110                         | 30                  | 10                  | 163                | 0.06                    |
|                             | 60                  |                     | 137                | 0.07                    |
|                             | 90                  |                     | 119                | 0.08                    |
|                             | 120                 |                     | 111                | 0.09                    |
| 120                         | 30                  | 10                  | 122                | 0.08                    |
|                             | 60                  |                     | 109                | 0.09                    |
|                             | 90                  |                     | 90                 | 0.11                    |
|                             | 120                 |                     | 109                | 0.09                    |
| 130                         | 30                  | 10                  | 130                | 0.07                    |
|                             | 60                  |                     | 125                | 0.08                    |
|                             | 90                  |                     | 129                | 0.07                    |
|                             | 120                 |                     | 134                | 0.07                    |

3.2 Quantification of antioxidants in green tea samples
The antioxidant activity of green tea was observed using µPAD obtained from the penetration temperature 120 °C and 90s penetration time. It was observed by the ability of antioxidant in reducing the FeCl₃. It was determined with the addition of 1, 10- phenanthroline as a colorimetric indicator (or based on colorimetric reactions). The color changes obtained from the samples were then compared with the color change of the standards (red green blue color intensity level of the standard was presented in Table 2.

Table 2. Red, green, and blue color intensity of standard

| Concentration (mg. L⁻¹) | R    | G    | B    | Δ Intensity (I₀ - I) |
|-------------------------|------|------|------|----------------------|
| 0                       | 251.111 | 244.112 | 232.858 | 0                    |
| 25                      | 249.917 | 219.898 | 220.911 | 1.194                |
| 30                      | 247.881 | 212.401 | 223.441 | 3.230                |
| 55                      | 242.517 | 202.518 | 218.764 | 8.594                |
| 75                      | 238.118 | 197.011 | 200.196 | 12.993               |
| 80                      | 236.211 | 188.102 | 190.232 | 14.900               |
| 100                     | 235.737 | 185.789 | 186.433 | 15.374               |
The samples tended to change the yellow color of FeCl₃ to an orange-red color, which is not affected by the acidity as explained by Szydlowska-Czerniak et al. [15]. The RGB intensity levels of the µPAD contained samples were shown in Figure 4. Figure 4 showed that the intensity value of red, green and blue increased but did not generate perfect linearity curves for the blue and green color. The red color changed level generated a smooth linearity curve, thus, it was used to determine the antioxidant activity of green tea. The RGB intensity level of both samples extracted in 25° and 90° was presented in Table 3.

**Figure 4.** Relationship between Concentration and Difference of RGB Intensity level

**Table 3.** Red Color Intensity Level of Green Tea Samples

| Sample      | Temperature (°C) | Δ Red Intensity |
|-------------|------------------|-----------------|
| Green Tea A | 25               | 1.597 ±0.4      |
|             | 90               | 8.475 ± 0.2     |
| Green Tea B | 25               | 2.161 ±0.6      |
|             | 90               | 3.344 ±0.3      |
| Green Tea C | 25               | 3.721 ±0.5      |
|             | 90               | 12.603 ±0.3     |
| Green Tea D | 25               | 3.449 ±0.3      |
|             | 90               | 14.072±0.5      |

The red intensity level of both standard and samples were compared. As compared to the standard (Table 2), green tea A has antioxidant activity at 25 mg. L⁻¹ and 55 mg. L⁻¹ for the 25° and 90° respectively. Green tea B had the antioxidant activity for 25° in between 25 and 30 mg. L⁻¹, meanwhile the 90° had 30mg. L⁻¹ antioxidant activity. Green tea C had 30 mg. L⁻¹ and 75 mg. L⁻¹ antioxidant activity for 25° and 90° extraction, respectively. Green tea D had the same antioxidant activity for 25° extraction as green tea C and 80 mg. L⁻¹ for the 90° extraction. The antioxidant activity of the samples
in two different extraction temperatures was presented in Figure 5. The temperature extraction, in this study, significantly affected the antioxidant activity of green tea which in accordance as explained by [16].

\begin{figure}
\centering
\includegraphics[width=\textwidth]{fig5.png}
\caption{Antioxidant Activity of Green Tea Samples at 25 °C and 90 °C}
\end{figure}

4. Conclusion
Antioxidant activity in green tea samples can be determined using the μPAD method based on redox reactions and formation of complex compounds with FeCl₃ and 1, 10-phenanthroline reagents. Acyl color intensity of the sample obtained compared with standard results obtained. Then the antioxidant results of green tea samples with infusion treatment at a temperature of 25 °C and 90 °C has a significant difference. The μPAD method can be used as a new alternative method for determining antioxidant activity in green tea samples that are cheaper, faster and easier to use.

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