Original Article

Folin-Chiocalteu colorimetric analysis using a scanner for rapid determination of total polyphenol content in many test samples

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Abstract

In order to develop a low cost method for rapidly determining the total amount of polyphenols in many samples, the colorimetric technique combined with the Folin-Chiocalteu (FC) method was investigated by using a digital scanner. Since the test solution containing polyphenols shows a blue-color after treatment of the FC method, the R-value was selected from RGB values of the digital image data obtained with a scanner. The absorbance which was calculated from the R-value was proportional to the concentration of a typical polyphenol such as chlorogenic acid and gallic acid. To discuss the cost performance of this colorimetric technique, the usual UV-Vis spectrophotometer method, the digital camera method and the micro-plate reader method were tested. As the linearity of a calibration curve obtained by the three methods was equivalent to that by the scanner method, the scanner method was decided to be the most effective technique from the standpoint of short measuring time and cost performance in the case of determination of the total polyphenol content in many samples. In the future, the FC-scanner method is likely to be multi-used at the site of functional food manufacturing.

Key words

colorimetric analysis, digital image, Folin-Ciocalteu method, polyphenol, scanner

1. Introduction

In recent years, phenolic compounds and derivatives, i.e. polyphenols are attracting extreme attention as one of functional compounds that prevent various diseases and anti-aging. The reason is due to the highly anti-oxidative effect of polyphenols against active oxygen species such as superoxide. When the anti-oxidative effect of a sample such as food is examined, it is important to identify the individual polyphenol compound by using a suitable method such as HPLC. In addition, the total polyphenol content in a sample is also thought to be an effective factor. In order to determine the total polyphenol content, the Folin-Chiocalteu (FC) method (Folin and Ciocalteu, 1927) and its modified techniques have been widely used by many researchers. In general, the absorbance of the blue-colored test solution treated by the FC method has been measured with a UV-Vis spectrometer. In this case, however, the method is thought to be unsuitable for the measurement of many samples in a short measuring time. To overcome this problem, recently, a digital camera, a digital scanner, and a micro-plate reader have been used elsewhere. (Aghanouri et al., 2010; Ishihara et al., 2008; Koga and Utsuoka, 2004; Koga, 2007, Kikuchi et al., 2011; Medina-Remon et al., 2009; Poce-Fatou et al., 2011)

In this study, these three methods will be compared from a standpoint of accuracy, the measuring time and the cost performance by measuring the calibration curve of the absorbance against the concentration of typical polyphenolic compounds. The digital images, which were obtained using a digital camera and a digital scanner, are treated with an RGB analysis, and the R-value is used to calculate the absorbance of a blue-colored solution. In the case of micro-plate reader, the absorbance is directly calculated because the light-source of fixed wave length is mounted. From all of the data, the best method will be decided. The digital scanner method is of a low cost and will make it possible to analyse a lot of samples in a short time by colorimetric analysis.

2. Materials and methods

2.1 Chemical reagents

Phenol reagent (PR) and sodium carbonate were purchased from Wako Pure Chemical Industries, Ltd. Japan. Chlorogenic acid (CA, 3-caffeoylquinic acid, $C_{16}H_{18}O_9$) and gallic acid (GA, 3,4,5-trihydroxybenzoic acid monohydrate, $C_7H_6O_5 \cdot H_2O$) were obtained from MP Biomedicals, LLC, Japan and Tokyo Chemical Industry Co. Ltd. Japan, respectively. All the reagents were used without further purification.

Each polyphenol of CA and GA was dissolved in deionized water at a concentration of 1 mg/mL. PR was diluted twice with deionized water. Sodium carbonate of 20 g/100 mL (20 %) was prepared with deionized water.

2.2 Instrumentation

A UV-Vis spectrometer (V630, JASCO Corporation, Japan) with a disposable cuvette cell was used to measure UV-Vis absorption spectra. The scanner (GT-S620, Seiko Epson Corporation, Japan) and digital camera (Nikon D70s, Nikon Corporation, Japan) were selected to obtain the digital image of test solutions. The micro-plate reader (ChroMate 4300, Awareness Technology Inc, United States) with 96-well micro-plate was also used to measure the absorbance (at 700 nm) of test solutions. The cell folder for the scanner was designed and made from an acrylic board, and then white acrylic spray paint was coated on the bottom of this folder as illustrated in Figure 1. Free software of GIMP from GIMP Org. (http://www.gimp.org/) was used to obtain the RGB values by image analysis.

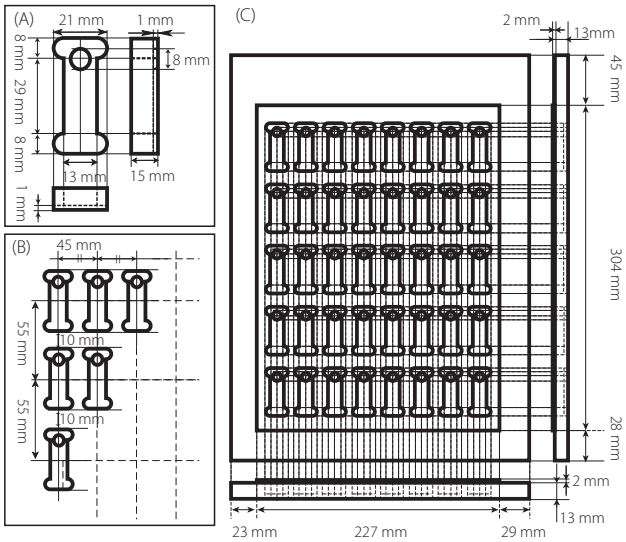

Figure 1: Overview of acrylic cell folder
(A) is the size of single cell, (B) is the interval of cells, and (C) is the overview of cell folder

2.3 Measurement of UV-Vis absorption spectra

Test solutions of concentrations of 0, 0.020, 0.040, 0.060, 0.080, and 0.100 mg/mL were prepared from the stock solution of CA with deionized water. Similarly, 0, 0.010, 0.020, 0.030, 0.040, and 0.050 mg/mL GA solutions were prepared. The solution of 0 mg/mL means deionized water without any reagent as a blank solution.

The measurement procedure is as follows. A polyphenol solution of 1 mL and twice-diluted PR of 1 mL were mixed in a disposable cuvette cell (4.5 mL). After 3 minutes, 1 mL of sodium carbonate solution was added. Ten minutes later, the cuvette cell was set in the cell folder of the UV-Vis spectrometer and the absorption spectrum of test solution was recorded at the range of 400 to 800 nm. From the spectrum, the value of absorbance was obtained at 760 nm.

2.4 Measurement using scanner

CA solutions of 0, 0.006, 0.012, 0.018, 0.024 and 0.030 mg/mL and GA solutions of 0, 0.005, 0.010, 0.015, 0.020 and 0.025 mg/mL were prepared with distilled water. CA or GA solution (1 mL) of each concentration, diluted PR (1 mL), and sodium carbonate solution (1 mL) were mixed in disposable cuvette cell by the same way described in the section of UV-Vis spectrometer. There are a few differences in this method.

All the cuvette cells, in which each test solution was contained, were set in the cell folder, and one scan was carried out. A target area was selected within the region of the cell in a digital image obtained, and an RGB-analysis was carried out using GIMP free software. The scanner was operated at the following conditions; no color calibration, resolution of 600 dpi and 64 bit color.

2.5 Measurement using micro-plate reader and digital still camera

The experimental methods using a micro-plate reader and a digital camera are highly similar. CA solutions of 0, 0.024, 0.048, 0.072, 0.096 and 0.120 mg/mL and GA solutions of 0, 0.014, 0.028, 0.042, 0.056 and 0.070 mg/mL were prepared from stock solutions. All of CA or GA solutions, PR, and sodium carbonate solution were mixed in 96-well cell. The point of difference from the other two methods described above is that the solution volume was reduced from 1 mL (1000 µL) to 100 µL. In the case of the micro-plate reader, the absorbance at 700 nm was automatically calculated using ChroMate Maneger equipped with ChroMate 4300. In the case of the digital camera, a micro-plate of 96-well was placed on the surface of a LED light panel in a darkroom, and a digital image was measured and analysed by the method similar to the scanner.

Figure 2 summarizes procedures described above.

2.6 Calibration curve of polyphenol

Calibration curves were obtained using the absorbance of each concentration of polyphenols (CA and GA) at 760 nm (V630) or at 700 nm (ChroMate 4300). On the other hand, GT-S620 and Nikon D70s cannot give absorption spectrum directly (Brian, 2000). Therefore, absorbance value (A) was calculated by means of Lambert –Beer's law of equation (1) from the R-value.

$$A = \log_{10}(I_0/I) \quad (1)$$

I means the incident and transmittance light intensity, and subscript 0 means a blank. In this study, I was replaced by R (R-value).

$$A = \log_{10}(R_0/R) \quad (2)$$

The solutions after FC treatment show the color of dark blue-green (cyan). This phenomenon means that the test solution absorbed red region light from incident light. Therefore,

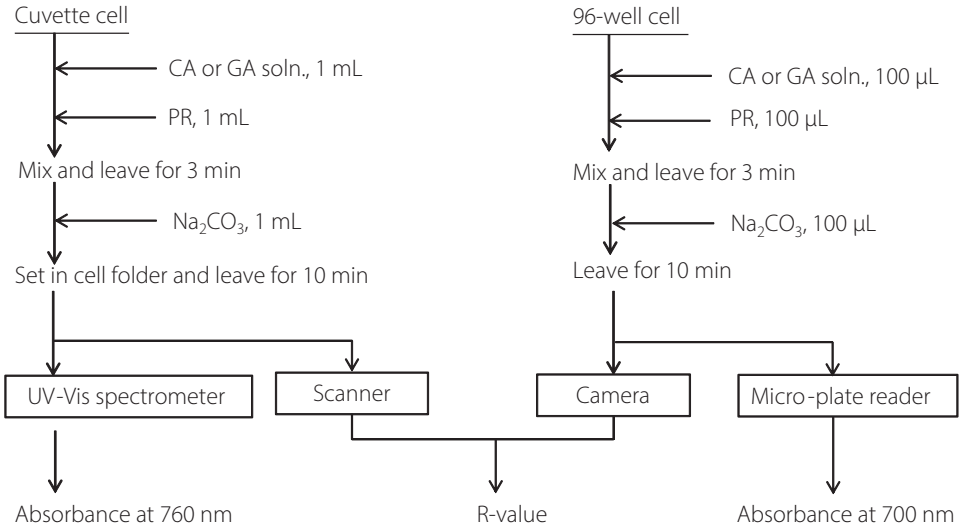

Figure 2: Experimental procedure

I_0 (or I) can be replaced by R_0 (or R).

3. Results and discussion

3.1 Decision of experimental conditions of FC method

When this study was started, the experimental conditions of the Folin-Ciocalteu (FC) method were decided. FC methods and the modifications have been reported elsewhere (Folin and Ciocalteu, 1927; Lowry et al., 1951; Anesini et al., 2008; Atanassova et al., 2011; Chandra et al., 2004; Everette et al., 2010; Fu et al., 2011; George et al., 2005; Hilal and Engelhardt, 2007; ISO, 2005; Kim and Wampler, 2011; Medina, 2011; Moraes et al., 2008; Paixao et al., 2005; Singleton and Rossi, Jr., 1965; Surveswaran et al., 2007; Tabata et al., 2008). Firstly, a test solution containing polyphenols is mixed with PR solution. After 2 to 5 minutes, sodium carbonate solution is added to the mixture. One to two hours later a calorimetric measurement is carried out. Many researchers have used PR solution of several concentrations that were diluted to 1/2 - 1/10 (1 N to 0.2 N), and used sodium carbonate aqueous solution of concentrations of 4 % to saturation.

Based on careful experiments, we used a 1/2-dilute solution (1 N) of PR reagent and 20 g/100 mL sodium carbonate solution. The mixture of the test solution and PR solution was left for 3 minutes before dropping 20 g/100 mL of sodium carbonate solution. During these experiments, it was found that the addition of 10 g/100 mL sodium carbonate generated the color gradation of the resulting solution and many bubbles of carbon dioxide. Therefore, it was necessary to remove this gradation and bubbles by stirring the solution with a glass rod. However, the addition of 20 g/100 mL solution presented a clear regular color without any bubbles. After the sodium carbonate solution was added, the solution was left 10 minutes and then colorimetric measurement was started.

Although the leaving time was 1 to 2 hours in previous literature, the leaving time of 10 minutes was found to be enough in the colorimetric measurement because the increase of absorbance was a little after one hour.

As a caution in the FC method, we denote the influence of ascorbic acid as an interfering substance. If a sample contains ascorbic acid, the measured absorbance includes the contribution of ascorbic acid because the acid is a reducing compound as well as polyphenol. In this case, we recommend a previous treatment with ascorbic acid oxidase.

3.2 Calibration curve using UV-Vis spectrometer

Figure 3 shows the UV-Vis absorption spectra of CA. Similarly, Figure 4 shows GA's one. Absorption of CA and GA increased with the increase in concentration of these polyphenols.

Figure 5 shows the calibration curves of the absorbance against the concentration of CA and GA. Both curves gave high correlation coefficients. Thus, the combination of the FC method and UV-Vis absorption spectrum is a useful technique to analyse total polyphenol content even if a modified FC method was applied. The slope of the curve of GA was larger than that of CA. If the concentration of polyphenol is calculated not by mg/mL but by mol/L, the difference will become

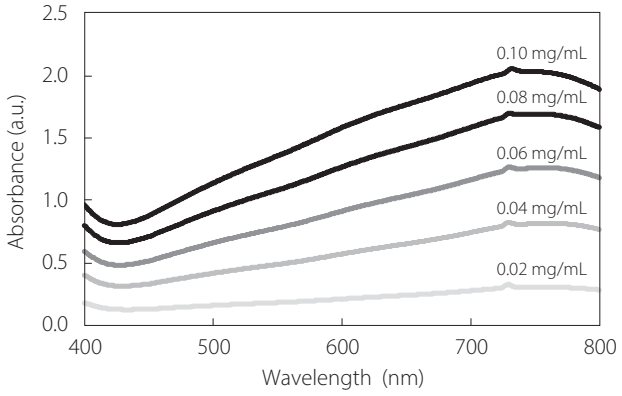

Figure 3: UV-Vis absorption spectrum of CA

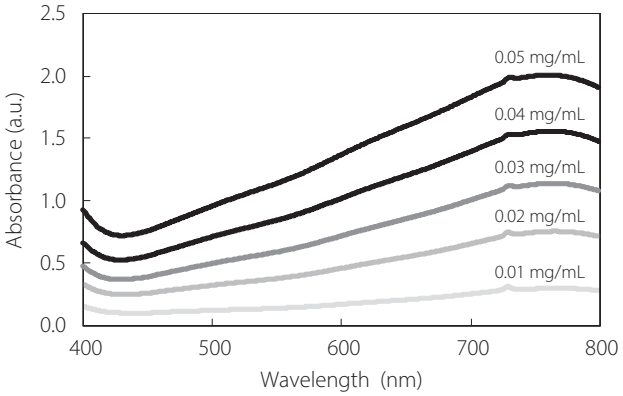

Figure 4: UV-Vis absorption spectrum of GA

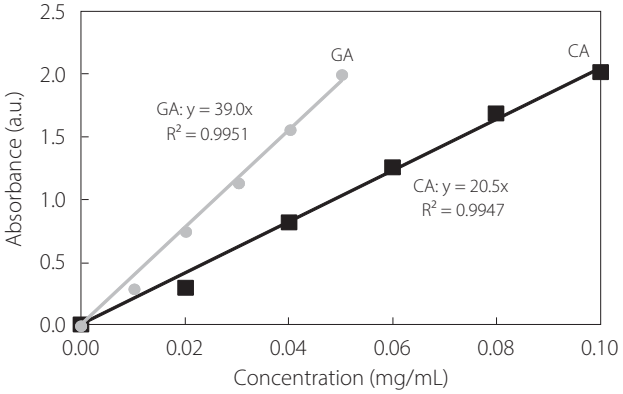

Figure 5: Calibration curve of CA and GA

smaller because the molecular weights are 354.3 and 170.1 for CA and GA, respectively. Of course, the amount can be expressed not only by mg/mL (or g/L) but also mol/L.

3.3 Calibration curve using scanner, micro-plate reader, and digital camera

Table 1 shows the experimental results of these three methods and the calibration curves are shown in Figures 6 to 8 with good correlation coefficients.

However, a cuvette cell and 96-well cell have different light path distance against the detector of each piece of equipment and also the UV-Vis spectrometer. Therefore, the slope

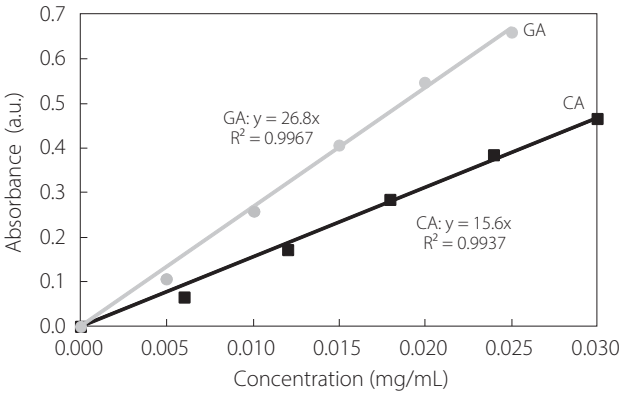

Figure 6: Calibration curve of CA and GA using the scanner (EPSON GT-S620)

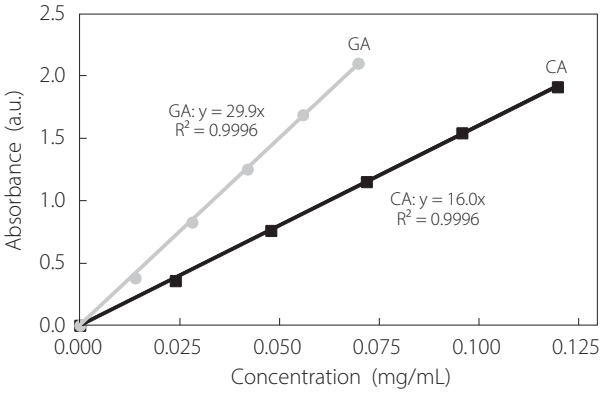

Figure 7: Calibration curve of CA and GA using micro-plate reader (ChroMate 4300)

Table 1: Absorbance data of CA and GA obtained by three methods

	Scanner (n = 9)				Micro-plate Reader (n = 9)			Camera (n = 3)			
	Conc.	$R_{Ave.}$	SD	Abs.	Conc.	Abs.	SD	Conc.	$R_{Ave.}$	SD	Abs.
CA	0.000	118	2.64	0.000	0.000	0.000	0.00	0.000	169	2.00	0.000
	0.006	101	1.90	0.068	0.024	0.357	0.02	0.016	137	2.52	0.090
	0.012	79	1.58	0.173	0.048	0.759	0.02	0.032	103	1.15	0.216
	0.018	61	1.54	0.287	0.072	1.160	0.01	0.048	74	1.00	0.359
	0.024	49	1.42	0.386	0.096	1.550	0.02	0.064	54	1.00	0.495
	0.030	40	1.13	0.466	0.120	1.920	0.01	0.080	39	1.00	0.637
GA	0.000	116	4.85	0.000	0.000	0.000	0.00	0.000	234	0.58	0.000
	0.005	91	3.66	0.107	0.014	0.387	0.02	0.010	188	0.00	0.096
	0.010	64	3.44	0.261	0.028	0.832	0.02	0.020	141	1.15	0.220
	0.015	45	3.88	0.409	0.042	1.260	0.03	0.030	105	0.58	0.350
	0.020	33	3.62	0.549	0.056	1.690	0.03	0.040	76	0.58	0.491
	0.025	25	2.55	0.662	0.070	2.100	0.04	0.050	53	1.00	0.646

Conc.; Concentration (mg/mL), $R_{Ave.}$; Average of R, SD; Standard Deviation, Abs.; Absorbance

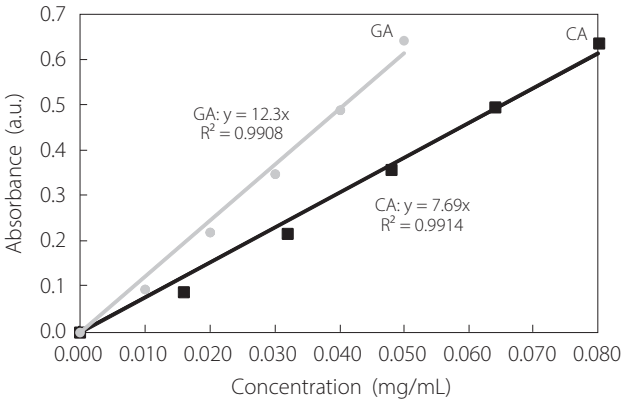

Figure 8: Calibration curve of CA and GA using digital camera (Nikon D70s)

is not equal to each other. As a reference, digital images are shown in Figures 9 (a) and (b) that were obtained from the scanner and the digital still camera, respectively. From these images R-values were calculated with GIMP software.

By the way, the linear range of absorbance in the calibration curve for UV-Vis spectrometer and micro-plate reader was not equal to that for the scanner and camera. The former showed the range of 0 to 2, but the latter the range of 0 to 1. At the range of 1.0 or more, the linearity of calibration curve decreased extremely (data is not shown). The reason is thought to be due to the essential limitation of an RGB analysis with 256 gradients.

3.4 Comparison of instrumentation cost

To compare the instrumentation cost, versatile tools such as a personal computer, micropipette and its tips, and photo retouch software such as GIMP and Photoshop, which are commonly possessed in laboratories and often are used for other experiments in a laboratory, were not considered. As shown in Table 2, the UV-Vis spectrometer is the most expensive but a scanner is the most economical. In the case that many samples are measured in a short measuring time, it can also be concluded that the scanner method is the most advantageous among the three methods except for the UV-Vis spectrometer.

4. Conclusion

As mentioned above, four colorimetric methods using a UV-Vis spectrometer, a scanner, a micro-plate reader and a digital camera were compared in order to determine the total polyphenol content by the FC method. From all the results, it is possible to recommend the colorimetric method using a scanner from the standpoint of short measuring time, low cost price, and measuring accuracy for many samples. The scanner method is possible to measure 40 samples simultaneously. In the future, the scanner method is likely to be multi-used at the site of functional food manufacturing.

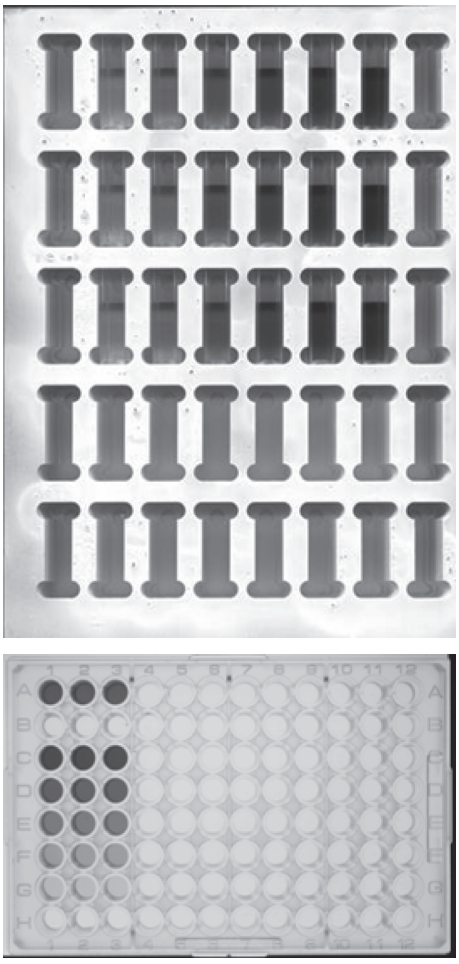

Figure 9: Digital image obtained from FC method
(a) GT-S620 image of FC-treated CA . (b) D70s image of CA (1-C to G, 2-C to G, 3-C to G), and unknown concentration samples (1-A, 2-A, 3-A). Micro-plate was placed on a flat LED light panel.

Table 2: Price list of equipment

Equipment	V630	ChroMate 4300	D70s	GT-S620
Price (JPY)	1200000	400000	100000	10000
Magnitude	100	33	8.3	0.83

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